1-1-2013

Role of iron in ethanol derived hepatic stress

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ROLE OF IRON IN ETHANOL DERIVED HEPATIC STRESS

A thesis submitted to

the Graduate College of

Marshall University

In partial fulfillment of

the requirements for the degree of

Master of Sciences

In

Biomedical Sciences

by

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August 2013

ACKNOWLEDGMENTS

I would like to begin this section first by saying that I've been fortunate enough to have a great advisor and mentor in Dr. John Wilkinson. He has been instrumental toward the development of my skills, confidence and critical thinking needed in this field and has always been understanding and supportive in the face of all the setbacks and successes; for that you have my gratitude. I'm also thankful for my committee members: Dr. Emine Koc for providing valuable feedback, which we owe for some very interesting discoveries made in this project and Dr. Vincent Sollars who helped with the experimental design, provided all the DNA methylation information, and helped me with the statistics. I would also like to think Dr. Monica Valentovic and her lab, who performed all the evaluations of SAMe and glutathione, as well as providing me with technical assistance. Last but not least, this project could not have been accomplished through the efforts of a few people. Feeding and weighing the mice on a nearly daily basis required a small army of graduate and undergraduate students who I would like to list by name: Kristen McKee, James Buchanan, Katie Bush, Rebekah Jamieson, Katy Curry, Trevor Stone, Kayla Chappelle, Ben Crowder, Jerrod Justice, and Whitney Loggins.

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LIST OF ABBREVIATIONS

4-HNE	4-Hydroxynonenal
AceCS2	Acetyl-Coenzyme A Synthetase 2
ADH	Alcohol Dehydrogenase
ALDH2	Acetaldehyde Dehydrogenase
CYP2E1	Cytochrome P450
FerH	Ferritin Heavy Chain
FerL	Ferritin Light Chain
GSH	Glutathione
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
IRE	Iron Response Element
IRP	Iron Response Element Binding Protein
LDL	Lebier-Decarli Liquid Diet
MAT	Methionine Adenosyltransferase
MS	Methionine Synthetase
NAD	Nicototinamide Adenine Dinucleotide

ROS	Reactive Oxygen Species
SAH	S-Adenosylhomocysteine
SAMe	S-Adenosyl Methionine
Sirt	Sirtuin (silent mating type information regulation)
Tf	Transferrin
TfR	Transferrin Receptor
THF	Tetrahydrofolate

ABSTRACT

Chronic alcohol abuse is the third leading cause of preventable death in the United States. Ethanol metabolism causes liver injury through alterations in hepatic metabolic state, redox status, and acetaldehyde adduct formations. Increased iron absorption is associated with chronic ethanol consumption and may play a role in ethanol induced oxidative stress. We tested the hypothesis that normal labile iron in the liver plays a role in ethanol related pathological stress, using C57/Bl6 mice pair-fed Lieber-DeCarli liquid ethanol diets for 11 and 22 weeks. Normal iron group mice received 55mg/kg iron as ferric citrate, whereas the low iron groups received 5mg/kg. Our findings indicate that chronic ethanol treatment did not result in discernible differences in oxidant stress from the controls as measured by 4-HNE residue formation and histological analysis of liver sections. Additionally, DNA methylation status was unchanged and there were no differences in glutathione and SAMe indicating no apparent impact on the methionine cycle. Analysis of hepatic proteins ferritin and transferrin receptor by western blot confirmed the expected response to the differences in dietary iron. Global protein acetylation was increased by ethanol treatment and further stimulated in animals fed the low iron diets. Overall, this study has identified novel interactions between iron deficiency and ethanol exposure.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Alcohol (ethanol) use is the third leading cause of preventable death in the United States in addition to being a major economic burden world-wide [1, 2]. It has been known that long-term ethanol consumption leads to changes in iron homeostasis that results in iron overload in the liver, leading to the possibility that iron may play a role in exacerbating the conditions that bring on alcoholic liver disease [3, 4]. However, little, to no attention has been given to how the interactions of ethanol and iron might change if dietary iron intake was decreased in chronic ethanol consumption, and more importantly whether these interactions might result in a pathophysiological change in the liver. My scientific interests lie in how chronic ethanol exposure and normal to sub-normal dietary levels of iron may interact through the generation of oxidant or other cellular stresses to generate pathological changes in the liver. Oxidative stress is thought to be a major contributor to many disease processes. Both iron and ethanol consumption have been independently linked to the generation of free radicals that cause oxidative stress [5, 6]. Chronic exposure to ethanol alone is known to cause tissue damage in the liver, impair other important metabolic processes, and increase the risk of cancer [6]. The purpose of this report is to test the basic hypothesis that dietary iron enhances ethanol-mediated liver injury. However, before considering their interactions, it is first necessary to understand the individual processes of iron regulation and ethanol metabolism in the liver. In this review I will present the most current

information concerning iron homeostasis and processes in the liver that are affected by ethanol metabolism including the transmethylation and transsulfuration pathways and global hepatic lysine acetylation.

1.2 Iron Homeostasis

Iron is an essential element that is important to many cellular processes including hemoglobin synthesis, enzymes involved in the electron transport chain, and providing reactive groups such as iron-sulfur clusters, etc. However, iron can be toxic when not bound to protein due to the ability of free iron to generate free radicals that cause oxidative stress. Particularly important to this process is the Fenton reaction (Figure 1), where divalent iron reacts with hydrogen peroxide to form hydroxide and the hydroxyl radical. When coupled with the ability of the superoxide anion to reduce trivalent iron to divalent iron, producing oxygen, the net reaction is called the Haber-Weiss reaction (Figure 1). Both because of the vital roles it fills and its ability to damage cellular components, the body has developed elaborate mechanisms to regulate iron both systemically and at the cellular level.

Fenton chemistry

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Haber-Weiss reaction

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^{-}$$

Figure 1. Iron generates oxidative stress. Ferrous iron generates free radicals through the Fenton and Haber-Weiss reactions.

Systemic Iron Regulation

The daily demand for iron is dominated by the 20-25mg that are required to maintain hemoglobin synthesis for erythrocyte production, as 65% of the iron found in the body is bound to hemoglobin [5]. The demand for iron is met primarily through the recycling of senescent erythrocytes by macrophages, but 1-2mg comes from dietary intake. Dietary non-heme iron is transported across the apical membrane of the intestinal duodenum via a specialized transporter protein. Non-heme iron exists mostly in the ferric form (Fe^{3+}) and must be reduced in order to be transported into the enterocytes. Duodenal cytochrome b (Dcytb), a ferric reductase reduces ferric iron to ferrous (Fe^{2+}) iron concurrently with transportation across the apical membrane. The absorption of heme bound iron is more efficient, and thought to occur through receptor mediated endocytosis [7-9]. Loss of iron from the body, aside from bleeding, is minimal, occurring only through the sloughing of intestinal mucosa and skin cells, and accounts for roughly 1 mg of iron per day. There are no known mechanisms for the excretion of iron.

When iron is needed, it is released from the basal membranes of intestinal enterocytes by the only known iron exporter protein ferroportin, through which iron enters into blood circulation. The iron quickly binds to transferrin (Tf) in the bloodstream, which can bind 2 molecules of Fe^{2+}. Tf-bound iron is shuttled through the portal vein and taken up by liver hepatocytes, facilitated by a type II membrane protein, the transferrin receptor (TfR) [7]. Because the liver is the primary iron storage site, it makes sense that systemic iron regulation is also controlled in the liver. Iron sensing proteins activate a hormone in the liver called hepcidin, through mechanisms that are

not well known [10]. Hepcidin regulates systemic iron by degrading ferroportin, effectively blocking iron release from enterocytes as and macrophages. When systemic iron is low, hepcidin is down-regulated to facilitate iron release from enterocytes, while the reverse applies in conditions of iron excess (Figure 2).

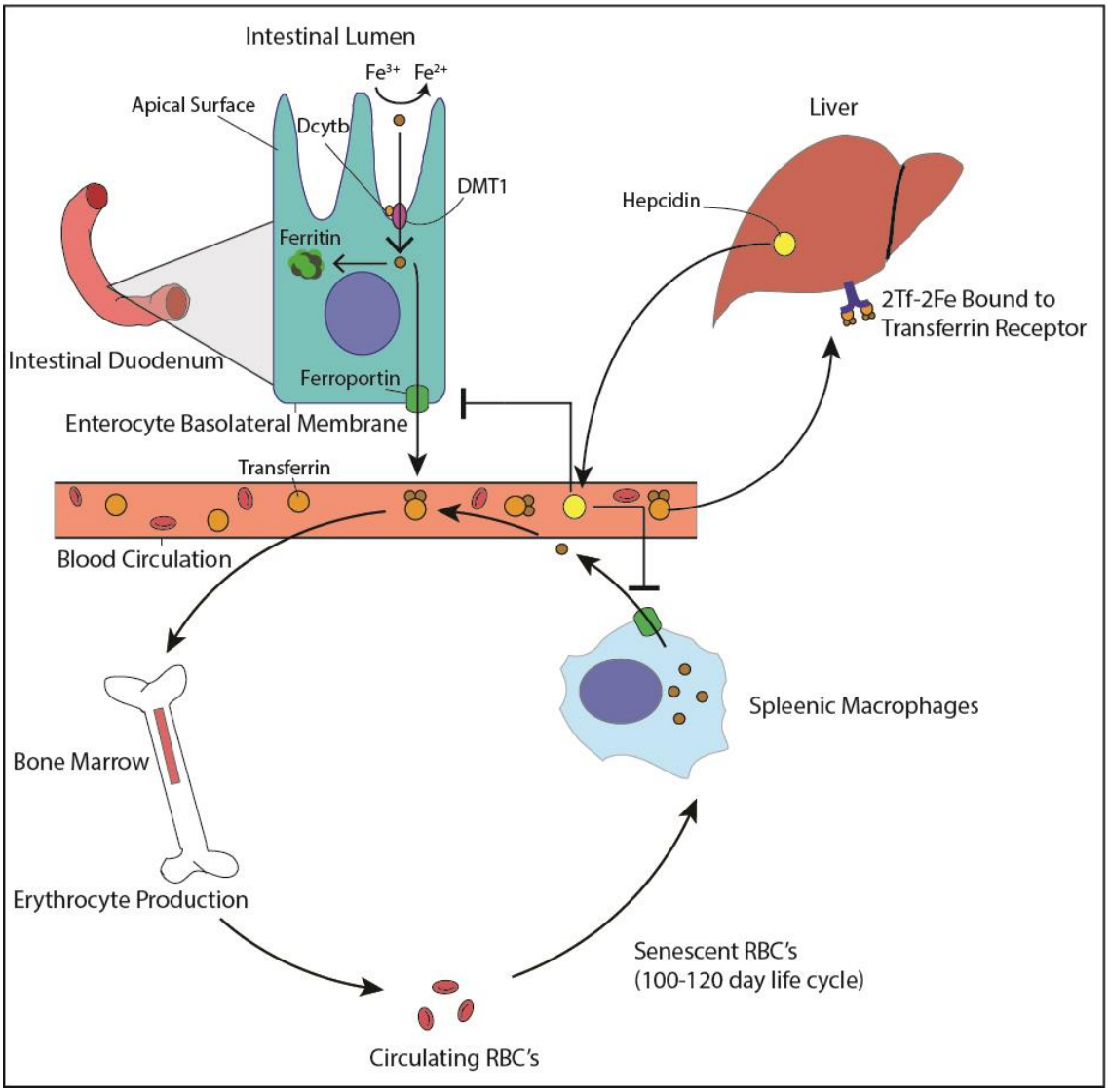


Figure 2. Systemic iron regulation. Dietary non-heme iron Fe^{3+} is reduced to Fe^{2+} by duodenal cytochrome b (Dcytb) before being taken up by duodenal enterocytes via the divalent metal transporter (DMT1). The iron is then either captured by ferritin or transported by the iron exporter protein ferritin and into the capillaries that drain the intestines. Iron is quickly bound to transferrin (Tf) in the blood which can bind two molecules of iron. Tf-bound iron is transported to the liver via the hepatic vein and taken up into hepatocytes by transferrin receptor (TfR). Tf-bound iron in circulation is also used for erythrocyte production in bone marrow. Circulating red blood cells (RBCs) have a lifespan of 100-120 days. The senescent RBCs are taken up by macrophages in the spleen where the iron is recycled and returned to circulation. Systemic iron is controlled by the liver produced hormone hepcidin, which is upregulated under conditions of iron excess. Hepcidin is released into the blood and degrades ferroportin in enterocytes and macrophages, effectively blocking iron release.

Intracellular Iron Regulation

Within various cell types there exists a transient pool of redox reactive, chelatable, free iron that is referred to as the labile iron pool. Labile iron is tightly regulated through post-transcriptional control of the expression of the transferrin receptor transport protein, and the ferritin iron storage protein (Figure 4). Ferritin is a 24 subunit oligomeric mixture of ferritin heavy (FerH) and light chains (FerL). The FerH subunit contains ferroxidase activity to convert cytosolic Fe^{2+} to Fe^{3+}, facilitating its storage within ferritin as ferric oxy-hydroxide phosphate [11]. Ferritin can sequester up to 4500 Fe^{3+} ions, which can be released from ferritin by proteosomal or lysosomal degredation [11].

The expression of TfR and ferritin is controlled by intracellular iron principally through post-transcriptional mechanisms involving iron response element binding proteins (IRPs) (Figure 3). A number of genes involved in iron metabolism, transport and storage contain iron response elements (IREs), which form hairpin structures in their mRNA to which activated IRPs can bind. The presence of iron de-activates IRP binding by increasing the affinity of the IRE binding site for iron-sulfur clusters; thus, when iron is high, more IRPs have a decreased binding affinity toward IREs. The location of the IRE in a gene dictates whether IRP binding inhibits translation (upstream location) or increases translation (downstream location). The two classic examples are transferrin receptor and ferritin. IRP binding, which occurs under low iron conditions, has the effect of increasing TfR translation by preventing the rapid degradation of TfR mRNA. Conversely, ferritin translation is decreased because the IRPs physically block the initiation of translation [11].

Thus, when iron is low TfR expression increases, allowing for the cellular uptake of iron to correct the balance, while ferritin expression is decreased, as iron storage is not needed when iron is low. During iron excess the reverse occurs: TfR expression decreases, while ferritin increases. These conditions are illustrated in Figure 4. Through examination of the steady state protein expression profiles of TfR and ferritin, one can determine the cell's iron phenotype, which may provide insight into how treatments impact intracellular and systemic iron.

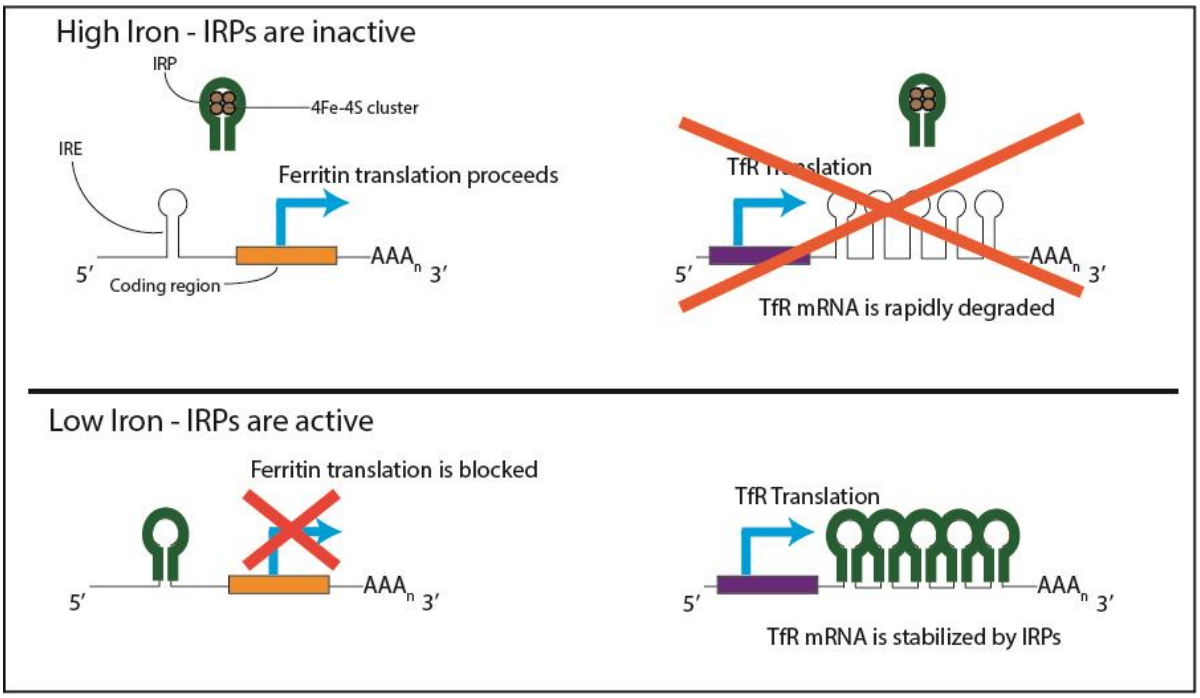


Figure 3. Post transcriptional regulation of ferritin and transferrin receptor. Under conditions of iron excess iron response element binding proteins (IRPs) become inactive due to the presence of iron increasing affinity of the iron response element (IRE) binding site for 4Fe-4S clusters. In this state ferritin translation is active and transferrin receptor (TfR) translation is decreased due to the rapid degradation of TfR mRNA. When iron is depleted, IRP's actively bind to the IRE's on mRNA. Ferritin translation is blocked by the action of IRP binding to upstream IRE's, while TfR translation is increased due to IRP's stabilizing TfR mRNA and preventing its degradation.

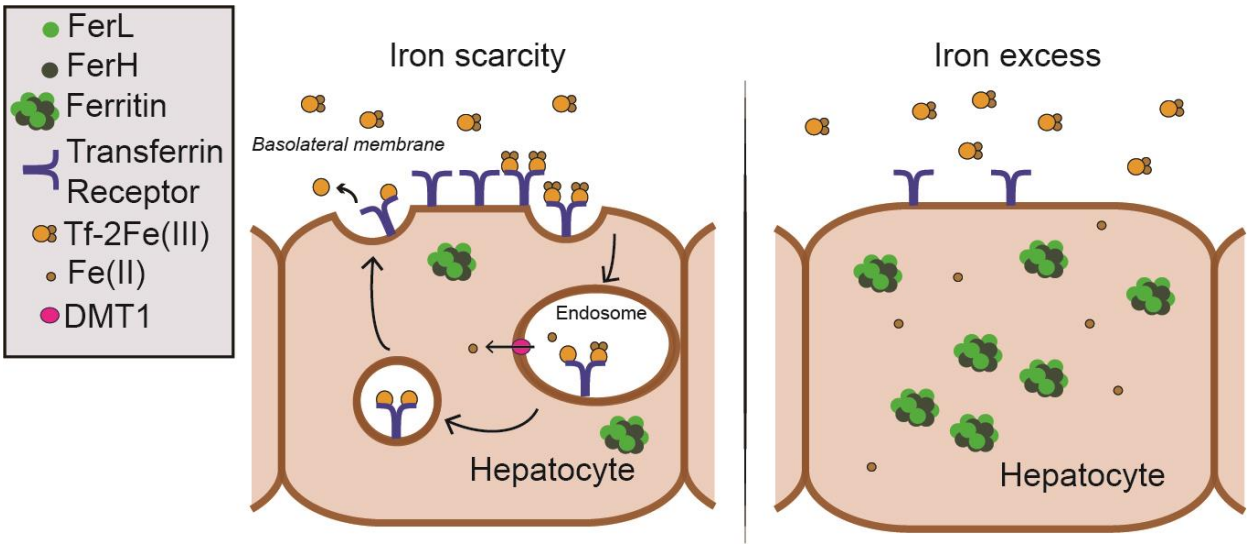


Figure 4. Intracellular iron regulation in liver hepatocytes. Low intracellular iron stimulates transferrin (Tf) receptor expression and down-regulates ferritin in order to facilitate the uptake of transferrin bound iron from blood circulation. Tf—2Fe is taken into the cell by receptor mediated endocytosis. The low pH in the endosome causes the release of iron from Tf, which is thought to be released into the cytosol by divalent metal transporter (DMT1). The Tf bound to TfR is returned to the cell membrane and Tf is released back into the blood. The iron storage protein ferritin, a 24mer consisting of subunits of ferritin light (FerL) and heavy chains (FerH), increases in response to high intracellular iron levels as transferrin receptor translation is repressed. Ferritin can also be up-regulated in response to oxidative stress which can arise from non-Tf bound ferrous iron (Fe^{2+}) in the cytosol.

Iron derived oxidative stress

Iron is an essential metal for life but its high reactivity is a double-edged sword because it can also be toxic, particularly when there is a disruption in iron homeostasis. Under normal physiological conditions most iron in the body is bound by protein [5]. For instance, the metal is usually found bound to Tf in blood circulation or captured by being stored in ferritin in hepatocytes [5]. However, iron overload can result in an increase of the labile iron pool, which is the primary source of iron derived oxidative stress in hepatocytes [12].

Free iron in the cytosol can react with hydrogen peroxide to generate hydroxyl radicals as indicated in figure 1. Fe^{2+} can react with lipid peroxides to generate lipid radicals as well. The reactive oxygen species (ROS) generated from these reactions can oxidize cell membranes, proteins and DNA, ultimately leading to apoptosis and other cell damage [5].

Iron overload is not typically a concern for most healthy individuals, although iron derived ROS may have an influence on the normal aging process [13]. It is mostly associated with genetic factors such as hereditary hemochromatosis. Individuals with hereditary hemochromatosis have a 200 fold risk of developing hepatocellular carcinoma due to excess iron deposition in liver hepatocytes [5]. An activity that is associated with phenotypic changes that correlate with iron overload is heavy alcohol consumption, which confers an increased risk of alcoholic liver disease [4, 14].

1.3 Ethanol Metabolism

When ethanol is consumed it diffuses across cell membranes quite readily and is absorbed directly from the stomach and small intestines. From there ethanol enters the bloodstream and is carried into the liver by the portal vein. Whereas other tissues in the body can metabolize ethanol, the primary site of metabolism is in liver hepatocytes [6]. Ethanol by itself is relatively harmless. However, highly reactive and toxic by-products are produced during the series of enzymatic reactions that occur in the liver to break down the alcohol [6].

Ethanol is metabolized by the liver in a series of oxidations to ultimately form acetate (Figure 5). In moderate alcohol consumers, ethanol is oxidized mainly in the cytosol of hepatocytes by alcohol dehydrogenase (ADH) to form the intermediate acetaldehyde. In heavy alcohol consumers about 10% of the ethanol is oxidized by cytochrome p450 2E1 (CYP2E1) in the microsomes and 2% can be utilized by catalase in the peroxisomes [4, 15]. Both alternate pathways also result in the formation of acetaldehyde. The acetaldehyde is further oxidized in the mitochondria by acetaldehyde dehydrogenase (ALDH) to form acetate. Acetate is activated by acetyl-coenzyme A synthetase 2 (AceCS2) in the mitochondria to form acetyl-CoA which is then utilized by the Krebs cycle to form NADH, CO_2 and water as end-products [16]. Alternatively, acetate can enter the bloodstream to be oxidized by mitochondria in other tissues such as the heart, skeletal muscles, and brain cells to also form NADH, CO_2 and water by the same pathways [6]. In each case, the NADH formed can be utilized by the electron transport chain to generate ATP [15].

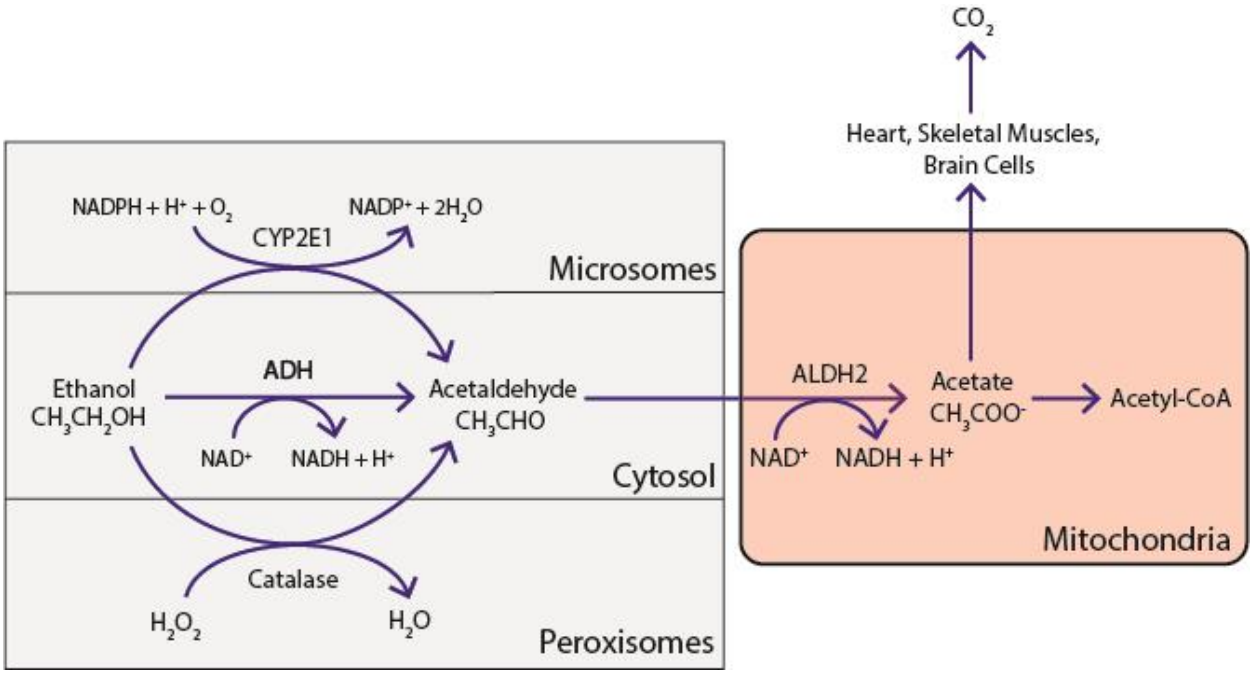


Figure 5. Ethanol metabolism overview in liver hepatocytes. Ethanol is oxidized primarily by alcohol dehydrogenase (ADH), or by catalase and cytochrome p450 (CYPE2E1) to form the toxic intermediate acetaldehyde. Acetaldehyde is further oxidized in the mitochondria by aldehyde dehydrogenase 2 (ALDH2) to form acetate, which can then enter the bloodstream or can enter the Krebs cycle in hepatocyte mitochondria to be completely oxidized to form CO_2 and water. ADH and ALDH2 both require nicototinamide adenine dinucleotide (NAD^+) to form NADH, which can alter the cellular redox state.

Ethanol alters the hepatic cellular redox state and energy metabolism

The oxidation of ethanol to acetate contributes to oxidative stress. The enzymes ADH and ALDH require the cofactor nicotinamide adenine dinucleotide (NAD^+) which is reduced to NADH in order to metabolize ethanol. The consequence of this is shifting the NADH/NAD^+ ratio, which changes the cellular redox state. Generally, the increase in NADH creates a more reducing environment in the cytosol and the mitochondria [15]. The redox active semiquinone intermediates within the electron transport chain complexes I and III in the mitochondria are also in a more reduced state due to the increase in NADH, facilitating the reduction of O_2 to O_2^-. Oxidative stress is also generated from NADPH oxidase activity which is required by CYP2E1 [15]. Since oxidative metabolism by CYP2E1 is NADPH depleting, it may negate the protection against oxidative stress that is conferred by glutathione (GSH) because NADPH is required in the regeneration of GSH from GSSG by glutathione reductase [17].

Although acetaldehyde is a short lived intermediate in the oxidation of ethanol to acetate, it is highly toxic and a known carcinogen. In addition to being a source of oxidative stress, acetaldehyde can readily form adducts with protein, enzymes, and DNA. The protein adducts formed can inhibit protein secretion, may contribute to enlargement of the liver, and cause mitochondrial dysfunction leading to cytochrome *c* release and activation of apoptotic factors [6, 15, 18]. As the mitochondria become impaired due to damage incurred from oxidative stress and acetaldehyde adducts, ATP generation is hindered as is the ability to process acetaldehyde, resulting in an accumulation of the carcinogen [15].

The accumulation of NADH has further deleterious effects that serve to alter energy metabolism. The high concentration of NADH inhibits the oxidation of lactate to pyruvate in gluconeogenesis and will cause the reverse reaction to predominate producing lactate [19]. The accumulation of lactate can lead to hypoglycemia and lactic acidosis [20]. Because fatty acid oxidation creates NADH to be used for ATP generation, the NADH glut inhibits β-oxidation of fatty acids, activating fatty acid synthesis instead [19, 21]. These metabolic changes sets events in motion that are consistent with the first stage of alcoholic liver disease, the development of fatty liver or hepatic steatosis through the accumulation of triglycerides [21].The second stage is alcoholic heptatitis, which is associated with cell death and inflammation [19, 22]. The last stage is cirrhosis as scar tissue develops around the dead cells [19].

The methionine cycle and transsulfuration pathway

The methionine cycle is important to the maintenance of methyl group donors that are used in transmethylation reactions (Figure 6). In a reaction catalyzed by methionine adenosyltransferase (MAT), methionine is converted to the methyl group donor S-adenosylmethionine (SAMe). SAMe serves as the principal methyl donor for a variety of important biological processes, such as epigenetic regulation of DNA and proteins, and is involved in the production of phospholipids and polyamines [23]. After the methyl group leaves, SAMe becomes S-adenosylhomocysteine which is hydrolyzed to adenosine and homocysteine. Homocysteine is used to synthesize glutathione (GSH), from cystathionine as part of the transmethylation pathway. Additionally, as an essential amino acid methionine cannot be synthesized but can be regenerated from homocysteine through dietary methyl group donors. The folate cycle provides methyl-

tetrahydrofolate (THF) which, through the action of methionine synthetase (MS) transfers a methyl group to homocysteine to generate methionine, thereby renewing the methionine cycle [24]. Collectively, we refer to the methionine cycle and the regeneration of methionine through folate biochemistry as one carbon metabolism.

Ethanol consumption has also been associated with decreases in SAMe/SAH ratios leading to (or and) an overall decrease in methylation, including DNA methylation [24]. Hypomethylation of DNA may result from ethanol's action at several points directly and indirectly involving one carbon metabolism. Ethanol has been shown to inhibit MS, restricting the ability to regenerate methionine from homocysteine [25]. There are many conflicting reports on how ethanol may decrease the activity of MAT, which would partially explain the decrease in methylation. One study has reported no changes in MAT level or activity were observed in rats intragastrically fed ethanol for 9 weeks. Despite this finding, liver biopsies from patients with alcoholic liver disease have shown decreases in MAT activity, suggesting that MAT may only be affected in later stages of alcoholic liver disease [24, 26]. Additionally, ethanol can interfere with vitamin B12 absorption [27, 28]. B12 is a required cofactor for the transfer of a methyl group from THF to homocysteine to form methionine, which is likely to disrupt the methyl donor pool. Should ethanol inhibit the methionine cycle and transsulfuration pathway, it is likely that GSH regeneration via transsulfuration derived cysteine would also be affected, decreasing the hepatocytes ability to scavenge free radicals, decreasing cellular protection versus oxidant stress.

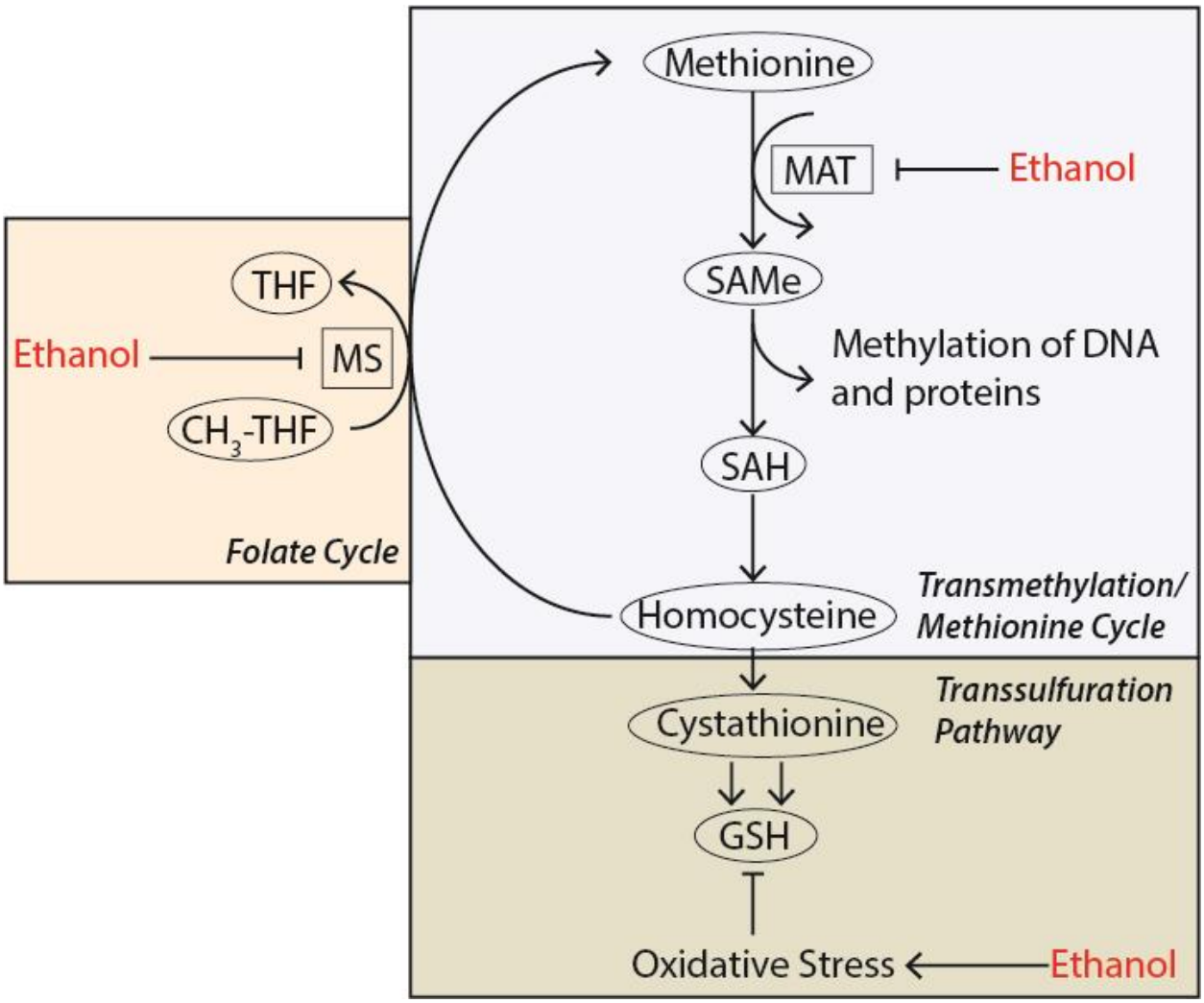


Figure 6. Ethanol perturbs the methionine cycle and transsulfuration pathway. The essential amino acid methionine is regenerated from homocysteine by the folate cycle which provides dietary derived methyl-tetrahydrofolate (THF). The methyl group is transferred to homocysteine to generate methionine by methionine synthetase (MS). Catalyzed by methionine adenosyltransferase (MAT) is converted to S-adenosylmethionine (SAMe), the primary methyl donator in the body. S-adenosylhomocysteine (SAH) is hydrolyzed to form homocysteine as one of the products, beginning the cycle again. Homocysteine is also used to synthesize glutathione from cystathionine. Ethanol exerts deleterious effects on these cycles by decreasing MS and MAT activity.

Lysine Acetylation

Lysine acetylation is a reversible post translational modification that has been studied and well characterized in the context of histone acetylation for over four decades [29]. However, the first non-histone protein to be identified as being modified by lysine acetylation, the tumor suppressor p53, has only come to light within the last two decades [30]. Since this discovery, the list of lysine acetylated proteins has been ever expanding. Proteomic studies have collectively identified at least 3,311 proteins with more than 7,151 lysine acetylation sites [31-33], many of which are enzymes that catalyze intermediate metabolism in the liver [32]. In general, lysine acetylation of histone tails is associated with transcription by neutralizing the lysine's charge and weakening the interactions between histones and DNA [34]. Understanding the function of lysine acetylation of non-histone proteins becomes more complex when the diversity of substrates are considered and may be analogous to phosphorylation in terms of the various ways in which proteins are regulated [35]. For instance, acetylation of axonemal tubulin is theorized to contribute to tubulin stability in nerve cells processes [36, 37]. Some of the key physiological mechanisms that are regulated by non-histone protein acetylation include cell migration, metabolism and aging as well as in the pathological context of cancer, neurodegenerative disorders and alcoholic liver disease [38, 39].

Lysine acetylation is catalyzed by the action of histone acetyltransferases (HATs). Despite the name, these enzymes modify both histone and non-histone targets; several have been identified and grouped into 5 subfamilies: HAT1, Gcn5/PCAF, MYST, p300/CBP, and Rtt109 [40]. HATs from the Gcn5/PCAF, MYST,

and p300/CBP families have all been detected in the liver and are identified to have mostly nuclear substrates. PCAF from the Gcn5/PCAF family is an exception which has been found to reside in both the nucleus and cytoplasm with substrates including histones, p53, and cortactin [39]. In prokaryotes and eukaryotes, lysine acetylation involves the transfer of an acetyl moiety from acetyl-CoA to the ε-amino group from an N-terminal lysine residue [41]. All HATs that participate in histone and non-histone acetylation contain a structurally conserved β-sheet helix core region that participates in acetyl-CoA binding in which the acetyl group serves as the donor for acetylation. The structurally conserved core region is flanked by variable N- and C- terminal segments that presumably contribute to substrate specificity [40]. Not much is known about how HATs are regulated, especially in regards to non-histone protein substrates. There have been many recent studies that show HAT activity in several families known to have nuclear substrates is at least enhanced by autoacetylation, but may not be a requirement for their activity [42-45]. These studies may provide insight into how non-histone protein acetylation is regulated as well. One such study has shown that a member of the Gcn5 acetyltransferase family associated with α-tubulin acetylation, αTAT1, has increased catalytic activity toward microtubules [46]. Other studies investigating lysine acetylation in Mycobacterium tuberculosis have shown that Gcn5-related acetyltransferases are regulated allosterically by cyclic AMP (cAMP) [47, 48].

The removal of an acetyl moiety from lysine residues on proteins is catalyzed by the better defined deacetylases. There are two major families of deacetylases, 11 belonging to the reduced potassium dependency 3/histone deacetylase 1 family (Rdp3/Hda1), generally referred to as the histone deacetylases (HDACs), and 7

belonging to the Sir2-related protein (sirtuin) family [49]. The two families of deacetylases are distinguished by their different catalytic mechanisms. HDACs utilize a highly conserved Zn^{2+}-dependent catalytic core, while Sirt activity requires NAD^{+} as a cofactor [49]. Although the catalytic mechanism of the HDAC's and Sirt's are fairly well characterized, not much is known about how they are regulated.

The deacetylases are further grouped into 4 classes. There are three of these classes in which HDACs are grouped based on size and homology to the yeast analogues Rdp3/Hda1 for HDACs, and a separate class for Sirts based homology to the yeast analogue sir2 [49]. HDAC1,2,3 and 8 are members of class I and are found almost exclusively in the nucleus with primarily histone and transcription factor substrates [39]. Class II consists of HDAC 4-7,9 and 10 with substrates that are mostly transcription factors. HDAC6 is the only exclusively cystosolic deacetylase and targets tubulin and Hsp90. Interestingly, HDAC6 knockout mice have hyperacetylated tubulin but develop normally with no distinct abnormal phenotype, although these mice had a slight decrease in bone mineral density [50]. Class III includes the sirtuins, sirt1-7. Sirt1 resides in the nucleus and substrates include histones and transcription factors such as p53, PGC1-α (peroxisome proliferator-activated receptor-γ coactivator), and the DNA repair factor Ku70. Each one of these is associated with cell survival, aging and caloric restriction [51-53]. Sirt2 regulates histones but also deacetylates tubulin and, like HDAC6, is cytoplasmic [54]. Sirt3-5 are found exclusively in the mitochondria [39]. Sirt6 and 7 reside in the nucleus and target H3K9 (histone 3 lysine 9) and RNA polymerase I, respectively [55, 56]. Currently, not much is known about the lone member of class IV, HDAC11.

Global hepatic lysine hyperacetylation is a recently discovered feature of ethanol exposure with numerous target proteins having been identified to date in an ever expanding list [57-59]. These proteins have been discovered to reside in all the major subcellular compartments including the cytosol, nucleus and mitochondria [39]. Currently, the overall consequences of protein hyperacetylation are still being explored. . Histone H3 hyperacetylation alters transcription and has been shown to up-regulate transcription of genes known to be up-regulated with ethanol consumption (ADH and glutathione S-transferase), while down-regulating genes known to be down-regulated with ethanol consumption (L-serine dehydratase and CYP 2C11) [39]. Ethanol induced acetylation of SREBP-1 and PGC-1α leads the enhancement of fatty acid synthesis [39]. Tubulin is hyperacetylated under ethanol exposure as well. This appears to lead to microtubule stability, while impairing protein trafficking [39, 60].

The mechanism of ethanol induced hyperacetyation is also under exploration. Certain experiments on hepatic cells *in vitro* in the presence of ethanol using inhibitors of ethanol metabolizing enzymes such as 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, and cyanamide, which inhibits aldehyde dehydrogenase have discovered that ethanol metabolism is required for hyperacetylation and that ethanol may need to be oxidized fully to acetate [60-62]. These studies have involved microtubules and histone H3, and no reports have explored how other proteins might respond to inhibition of ethanol metabolism. If similar results were found from such experiments it would support a role for acetate or acetaldehyde mediating ethanol induced acetylation. Another hypothesis is that the decrease in NAD^+ due to ethanol metabolism, down-regulates sirtuin activity which requires NAD^+ as a cofactor for

activity [39]. There has also been evidence for decreased expression of HDAC6, Sirt1, and Sirt5 with ethanol exposure [58, 63, 64].

Interestingly, many of the proteins identified to be hyperacetylated with ethanol exposure have been found to reside in the mitochondria [57, 59]. And even though Sirt3 is thought to be the primary deacetylase in the mitochondria, Sirt3 levels were unchanged in one study using rats fed an ethanol diet consisting of 36% kcals derived from ethanol for 6 weeks [59]. However, it has also been shown that Sirt3 can be inhibited by covalent 4-HNE modifications, which may provide an alternative explanation for ethanol-derived increases in mitochondrial protein acetylation [63]. To date, there has been no acetyltransferase identified in the mitochondria which has led to speculation of a possible non-enzymatic mechanism of acetylation [65]. It has been shown that Sirt3 activity can deacetylate succinate dehydrogenase flavoprotein, a subunit of complex II an important component of the electron transport chain, leading to increased activity in complex II [66]. This finding implies that if Sirt3 activity is impaired with ethanol consumption, this could potentially lead to decreased activity of complex II and hinder oxidative phosphorylation.

Research in the area of ethanol induced hyperacetylation is still in its infancy and much more work needs to be done toward characterizing both the mechanisms and pathophysiological consequences of this observed phenomenon.

CHAPTER 2

ROLE OF IRON IN ETHANOL DERIVED HEPATIC STRESS

2.1 Introduction

Throughout human history, alcohol has been the most socially accepted addictive drug worldwide. It is estimated that alcohol consumption is the third leading cause of preventable death in the United States alone while globally accounting for up to 4% of total mortalities [1, 2]. Chronic alcohol use is associated with a number of diseases including Alzheimer's disease, liver disease, chronic respiratory disease, heart disease, diabetes, bone disease, and cancer [18].

Ethanol (CH_3CH_2OH) is the form of alcohol that is ingested and the primary site of metabolism occurs in the liver, which is the organ most directly affected by chronic alcohol use [6]. Ethanol is oxidized in a series of reactions in the liver, first producing the toxic intermediate acetaldehyde by ADH (alcohol dehydrogenase) in the cytosol, requiring the reduction of NAD^+ to NADH. Then, acetaldehyde is shuttled into the mitochondria and oxidized by ALDH2 (aldehyde dehydrogenase), also requiring NAD^+, to form acetate [6]. These reactions alter the cellular redox state and energy metabolism in liver hepatocytes and can lead to alcoholic liver disease. The first stage of which develops as hepatic steatosis, or "fatty liver" and with continued chronic alcohol abuse can progress to cirrhosis of the liver [19].

Pathogenesis due to alcohol use arises from a range of sources, all derived from the consequences of ethanol metabolism. Oxidative stress is thought to be a major contribution to liver injury. ROS (reactive oxygen species) can be generated from

acetaldehyde, changes in the cellular redox state, and metabolism of ethanol by CYP2E1 (cytochrome p450), an alternative cytosolic pathway that engages under high levels of cellular ethanol, and which is induced following chronic alcohol use [15, 18]. A key indicator of oxidative stress is the presence of 4-HNE (4-Hydroxynonenal) adducts that attaches to proteins, formed as a result of lipid peroxidation [67].

Ethanol also has an inhibiting effect on the transmethylation pathway, impacting the regeneration of methionine in the methionine cycle [24]. When this pathway is inhibited it reduces the amount of the methyl donor SAMe (S-Adenosyl methionine) available for reactions requiring methyl groups, such as DNA methylation [24, 26]. Additionally,impacts on the methionine cycle may affect synthesis of the ROS scavenger glutathione through the transsulfuration pathway, which requires homocysteine. We speculate that, conversely, ROS derived from ethanol consumption may lead to glutathione depletion and subsequent reduction in SAMe methyl donor levels and downstream methyl donor dependent reactions, such as DNA methylation, as occurs in Syrian hamster liver cells following bromobenzene treatment [68].

Recent studies have shown that chronic alcohol consumption also induces global lysine hyperacetylation of proteins [39]. Dozens of proteins have been identified as substrates for hyperacetylation under ethanol exposure that are nuclear, cytosolic and mitochondrial in origin [57, 59]. The mechanism underlying the increase in acetylation is currently unknown, although there have been reports of decreases in deacetylase activity associated with ethanol exposure, such as Sirt1 (silent mating type information regulation) and HDAC6 (histone deacetylase) [58, 69]. It has also been proposed that

the NAD^+ (nicotinamide adenine dinucleotide) dependent family of deacetylases, sirtuins, are down regulated because ethanol metabolism depletes free NAD^+ [39].

Iron is a known mediator of oxidative stress, generating ROS as a product of Fenton chemistry in which free iron reacts with hydrogen peroxide to form the hydroxyl radical [5]. Iron is taken in from the diet and absorbed in the small intestines, where it enters the blood stream, binds to transferrin and is collected in the liver via TfR (transferrin receptor) activity [7]. Iron in the liver that is not being utilized by biological processes, such as hemoglobin synthesis, is primarily stored in a non-reactive state within the ferritin protein, a 24 subunit oligomeric mixture of ferritin heavy chain (FerH) and light chain (FerL) subunits [5]. Systemic iron availability is regulated by a small peptide hormone produced by the liver called hepcidin. When up-regulated by increased iron stores, hepcidin works by triggering the degradation of the iron exporter protein, ferroportin, effectively inhibiting release of enterocyte-absorbed iron into the bloodstream [7]. Chronic alcohol use is known to inhibit hepcidin and desensitize the regulation of its expression to iron levels, leading to increased iron absorption and hepatic iron overload, one of the hallmarks of alcoholic liver disease [3, 70, 71]. The risk of liver disease ranging from cirrhosis to hepatocellular carcinoma is greatly increased in iron overload disorders such as hereditary hemochromatosis, an autosomal recessive disease in which excess iron is absorbed and deposited in the liver hepatocytes [72].

In the present study, we were interested in exploring the role of normal dietary iron levels in the hepatic stress that derives from chronic ethanol consumption. While many studies have examined this area in the context of iron overload, we were

interested in determining the impact of reducing dietary iron in ethanol fed mice to give insight into the role of normal iron levels in ethanol-derived liver damage. We hypothesized that lowering the amount of dietary iron would reduce oxidative stress in the liver resulting from chronic ethanol consumption, reducing in turn, the impact of ethanol on methyl donor availability. Thus, in addition to oxidative stress measurements we have explored the impact these diets have on methylation while maintaining normal, adequate levels of methyl donors in the diet. Finally, we evaluated their effect on global hepatic acetylation and the NAD^+/NADH pool to determine if iron availability is involved in ethanol's impact in these areas. We found no detectable involvement of oxidant stress after our chronic ethanol treatment, mild pathological changes to the liver, and interesting increases in hepatic protein acetylation in ethanol treated mice fed low iron diets when compared to ethanol treated mice receiving normal dietary iron.

2.2 Materials and Methods

All animal protocols were approved by the Marshall University Institutional Animal Care and Use Committee. C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, Maine) were housed in a AAALAC accredited facility and pair-fed Lieber-DeCarli liquid diets for 11 weeks and 22 weeks as previously described in the literature differing only in the amount of iron in the diet [73]. The Lieber-DeCarli diets were purchased from Dyets, Inc (Bethlehem, PA). The mice were divided into two experimental groups, normal iron and low iron, with both groups having ethanol and pair-fed control mice. The ethanol diets consisted of 35% calories from fat, 17% from

carbohydrate, 18% from protein, and 30% from ethanol with nutritionally adequate amounts vitamins, minerals, and methyl donors such as folate and choline. Control mice received an isocaloric amount of carbohydrate in the form of maltose-dextran. Mice fed a normal iron diet received 55mg/kg of ferric citrate, while the low iron diet delivered 5mg/kg of ferric citrate.

After a period of 11 weeks to 22 weeks of feeding, the mice were weighed and then euthanized by exsanguination. Hematocrits were measured and excised livers were washed in PBS-T, weighed and frozen in liquid nitrogen. The tissue samples were then stored in a -80ºC freezer. Liver sections were preserved for histology analysis in buffered formalin.

Liver tissue lysate preparation

Liver tissue was homogenized in whole cell lysis buffer (WCLB) containing 25mM tris pH 7.4, 1% triton X100, 6% SDS, 1% Na deoxycholate, 150mM NaCl, 200mM EDTA, and HALT protease inhibitor cocktails from Thermo Scientific (Rockford, IL). Approximately 15µL of WCLB was used per 1mg of tissue. Liver tissue was taken from -80°C freezer storage, weighed and ground with 65% of the total amount of WCLB to be used into fine particles using a pre-chilled mortar and pestle over liquid nitrogen. While still frozen the particulate matter was transferred into a 10mL polypropylene round bottom tube. The remaining 35% of WCLB was added to the ground tissue and it was homogenized on ice with a Tissuemiser polytron blender from Fisher Scientific (Fairlawn, NJ). The homogenate was centrifuged for 10 minutes, 11,000 RPM, at 4°C and the supernatant was collected.

Total protein was quantitated using the BCA protein assay kit obtained from Thermo Scientific (Rockford, IL). Western blot loading stock with a concentration of 5µg/uL was prepared by diluting lysate in modified 1X Laemmli sample buffer solution at a final concentration of 2% SDS, 10% glycerol, 63mM tris pH 6.8, 5% β-mercaptoethanol, and less than 0.05% bromophenol blue [74]. The samples were then boiled in water for 10 minutes and slowly cooled to room temperature for at least 30 minutes. Aliquots of stock were made to avoid numerous freeze/thaw cycles, then stored along with any unused portion of homogenate at -80°C.

SDS-PAGE

Samples were analyzed using SDS-PAGE with the mini-PROTEAN 15-lane gel system from Bio-Rad (Richmond, CA). The gels were cast using 12% polyacrylamide resolving gels (0.6M tris pH 8.8, 12% acrylamide/bis 37.5:1, 0.1% SDS, 0.05% APS, 0.1% TEMED) and 5% stacking gels (0.01M tris pH 6.8, 5% acrylamide/bis 37.5:1, 0.1% SDS, 0.05% APS, 0.1% TEMED). Prior to loading on the gel, the samples were prepared from western blot loading stock, that was previously frozen at -80°C, to the desired amount of protein to be loaded and boiled for 2.5 minutes. Gel running buffer was made from a 10X stock solution (1X = 0.3%(w/v) Tris, 1.44%(w/v) glycine, 0.02%(v/v) SDS). Electrophoresis was performed for approximately 1 hour at 200V, 125mA, and 12W until the dye ran off the gel.

Western Blots

The proteins were transferred using a wet transfer system, the Mini Trans-Blot Cell from Bio-Rad (Richmond, CA), on to Whatman Protran 0.1µm pore size

nitrocellulose membranes purchased from GE Healthcare (Little Chalfont, United Kingdom). The transfer buffer used was made from a 10X stock solution of Tris and glycine. Transfers were carried out at 4°C for 16 hours using the following power settings: 30V, 90mA, 25W. Even transfer and equal loading of proteins was evaluated by Ponceau-S staining the membranes (5%(v/v) acetic acid, 0.1%(w/v) Ponceau-S).

Following transfer and staining, the membranes were blocked for one hour in a solution containing 5%(w/v) powdered milk and 2%(w/v) bovine serum albumin (BSA) in 1X TBS-T made from a 10X stock solution (1X = 100mM tris, pH 7.4, 0.9%(w/v) NaCl, 0.05%(v/v) tween). The membranes were either washed in TBS-T for 5 minutes and probed immediately with antibodies, or dried and stored at -4ºC until needed. If the membranes were dried, they were blocked again before use for 15 minutes, washed with TBS-T for 5 minutes and then probed. All primary antibodies were incubated with gentle rocking overnight at 4ºC. Next, the membranes were washed at room temperature in TBS-T 1 time for 1 minute, 2 times for 5 minutes, and 2 more times for 15 minutes. Secondary horse radish peroxidase (HRP)-conjugated antibodies were incubated with the membranes at room temperature for one hour with gentle rocking, then washed with TBS-T for 1 minute, and 4 times at 5 minute intervals. HRP is activated by Super Signal West Pico chemiluminescent substrate purchased from Thermo Scientific (Rockford, IL), with 5 minutes incubation time. Lastly, the membranes were exposed to 8x10 clear blue x-ray film obtained from Thermo Scientific, using a Kodak Biomax cassette (Rochester, NY). The film was developed in a Mini Medical series x-ray film developer by American Medical Imaging Solutions (Huntington, WV).

Densitometry was performed using Un-Scan-It graph digitizing software purchased from Silk Scientific Inc. (Orem, Utah).

Antibodies

All antibodies were incubated in TBS-T, the 4-HNE antibody being the exception as it required a dilution in 5% milk, with dilution factors determined empirically. Additionally, all antibody dilutions were confirmed to have the ability to distinguish a linear concentration of protein amounts.

The following primary antibodies were used: custom rabbit α-mouse FerL corresponding to amino acid sequence Ac-EGVGHFFRELAEC (New England Peptide, Gardner, MA), mouse α-human TfR (Invitrogen, Camarillo, CA), goat α-4-HNE (Millipore, Billerica, MA), rabbit α-acetyl lysine (Immunechem, Burnaby British Columbia, Canada); rabbit α-mouse HDAC6, chicken α-mouse Sirt1 and rabbit α-mouse Sirt2 were obtained from Thermo Scientific (Rockford, IL).

The corresponding secondary antibodies were purchased from: Bio-Rad (Richmond, CA) goat α-mouse IgG-HRP, goat α-rabbit IgG-HRP, Thermo Scientific (Rockford, IL), donkey α-goat IgG-HRP Santa Cruz Biotechnology (Dallas, TX), and rabbit α-chicken IgY-HRP GeneTex (Irvine, CA).

HPLC Analysis of Hepatic SAMe Levels

A 200 mg aliquot of liver was homogenized on ice in 0.4 mM $HClO_4$ and adjusted to a final 1 ml volume. Mitochondrial and 15,000 x g supernatant suspensions were added to an equal volume of 0.4 mM $HClO_4$ to precipitate protein. Nuclear samples

were concentrated by lyophilizing the 1 ml of sample (total liver weight 600-900 mg) and reconstituting the sample in 125 µl 0.4 mM HClO4. The samples were then centrifuged at 10,000 x g at 4°C and filtered through 0.45 µM Millex®-HV filters (Millipore; Billericia, MD). A 20 µl sample of the filtrate was analyzed for whole liver, mitochondrial, and 15,000 x g supernatant fractions, while 40 µl of sample was required for detection of nuclear SAMe levels. SAMe and SAH levels were detected using a Beckman Coulter HPLC system (Fullerton, CA) with a 126 Solvent Module and a 166 Variable Wavelength Detector. The column was a YMC ODS-AQ 3 µm 120 Å 4.6x150 mm column. The mobile phase was a gradient at a flow of 1 ml/min (Waters Corporation; Milford, MA). The mobile phase gradient program was 8 minutes of 90:10 A:B followed by 12 minutes of 60:40 A:B. Mobile phase A consisted of 8 mM 1-heptane sulfonic acid sodium salt and 50 mM sodium phosphate monobasic (pH 3). Mobile phase B was 100% HPLC grade methanol. The wavelength for detection was 254 nm.

Determination of Hepatic Glutathione.

GSH was determined using a spectrophotometric assay. For the assay, 200 mg of liver was homogenized in 5% sulfosalicylic acid (SSA) for a final volume of 1 mL. GSH was assessed using glutathione reductase with 5,5'-dithiobis(2-nitrobenzoic acid) and NADPH. GSSG was measured by derivitizing the samples with 2-vinylpyridine first allowing determination of a percentage of oxidized glutathione within the cell.

NAD^+/NADH assay

NAD^+ and NADH levels were measured using the NAD^+/NADH quantification kit purchased from Biovision (Milpitas, CA). 4-5mg of liver tissue were taken from -80ºC

storage and homogenized with the polytron blender using the buffer provided by the kit. Following homogenization of tissue, the samples were clarified by centrifugation at 4ºC at 11,000RPM for 30 minutes, collecting the supernatant. Aliquots were taken from the supernatant to determine total protein amounts, and the remaining supernatant was immediately filtered using Amicon Ultra 10kDa centrifugal filters, purchased from Millipore (Billerica, MA), to remove any NAD degrading enzymes. The assay was carried out on the resulting filtrate following the kit instructions.

Statistics

Statistics were generated using one way ANOVA in Microsoft Excel and two tailed t-tests were conducted where appropriate. P-values were adjusted using the Bonferroni-Holm correction method to determine significance.

2.3 Results

Our purpose was to determine if alteration of liver iron would alter the ability of ethanol to impact several endpoints in the liver. We fed mice Liber-DeCarli based liquid diets (with nutritionally adequate levels of methyl donors) to deliver 55mg/kg of ferric citrate in the normal iron dietary formulation and 5mg/kg in the low iron diet. Ethanol concentrations in the diet were steadily increased by 2.5% of Kcals every half week until they reached 30%. Pair-fed controls received the specific number of calories and equal nutrients as ethanol fed mice, by replacing the ethanol derived calories with maltose-dextran.

Mice were purchased from the Jackson Laboratories at 10 weeks of age, and allowed to acclimatize to basal normal iron liquid diet for one week prior to starting the

experiment. The first wave groups (see Table 1) were seeded with 4 mice in each group. The second and third subsequent waves were also seeded with 4 mice per group. The groups within each wave started on the respective diets at the same time. Wave 1 mice were fed experimental diets for 11 weeks, and then weighed, euthanized and tissues were taken at necropsy. Wave 2 and 3 mice were fed experimental diets for 22 weeks, and then weighed, euthanized and tissues were taken at necropsy. Hematocrits were measured on the blood drawn from mice; no differences were observed (Supplementary Figure 13).

	11 Weeks of ethanol exposure		22 weeks of ethanol exposure	
Treatment Group	**Normal Iron**	**Low Iron**	**Normal Iron**	**Low Iron**
Ethanol	**4**	**4(3)**	**8**	**8(7)**
Pair-Fed Controls	**4**	**4(3)**	**8(7)**	**8(7)**

Table 1. Overall study design. Indicated in the table is the number of mice seeded per group in each wave. In parenthesis is the number surviving the experiment.

Liver Pathology

In order to evaluate the pathological impact of the ethanol diets histological sections of liver were prepared and examined; all samples were found to have mild steatosis, but no progression beyond this stage. Body weights of mice were noted as well as the liver/body weight ratios as indicated in Figure 7. There were no significant changes in body weight at 11 or 22 weeks between diet groups. However, on average there was an approximate 30% increase in liver/body weight ratios in ethanol fed mice compared to their respective controls at 11 weeks and 22 weeks

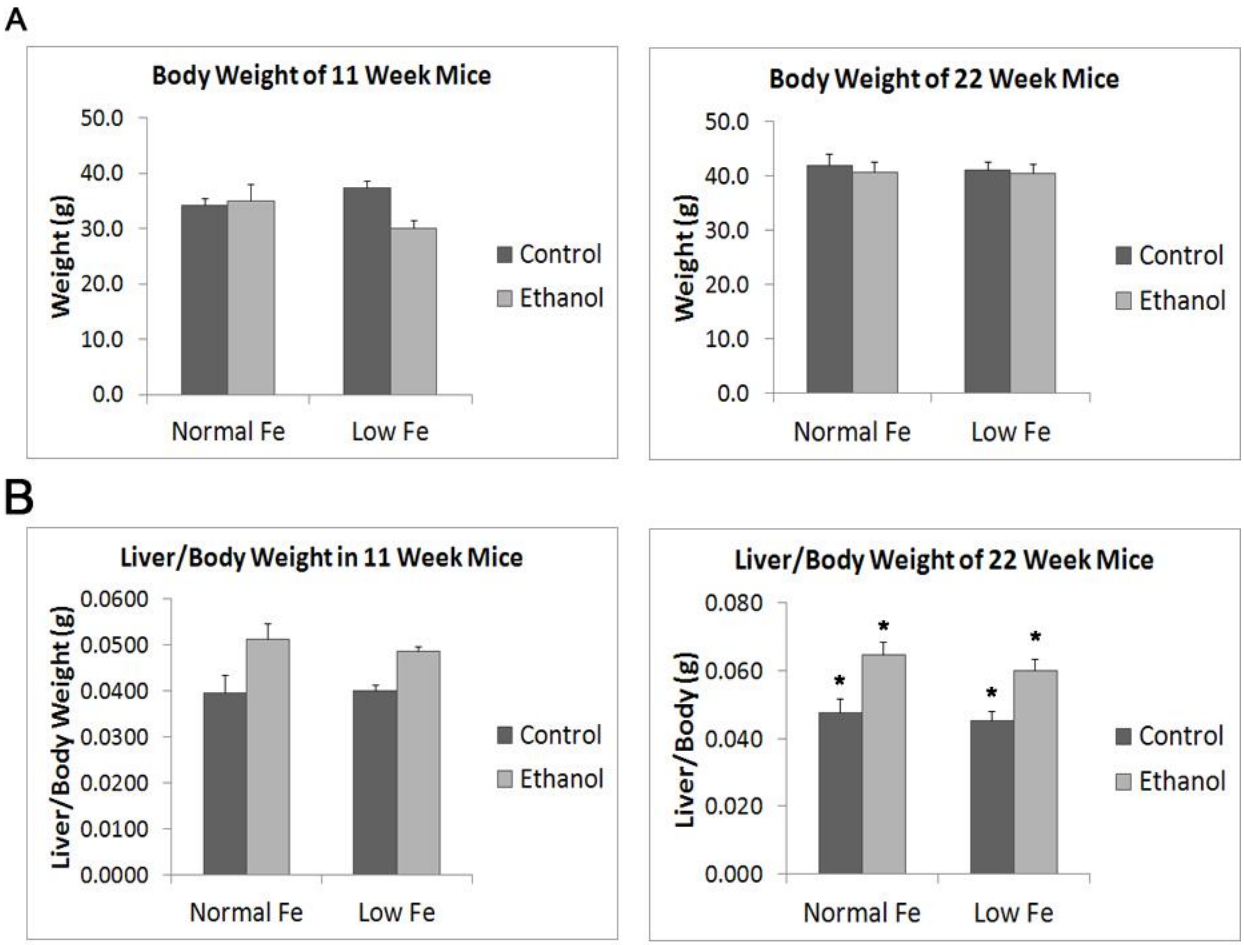


Figure 7. Mouse weight and liver/body weight ratios at time of necropsy. (**A**) Body weights of 11 and 22 week ethanol fed mice. (**B**) Liver/body weight ratio of 11 and 22 week ethanol fed mice. Error bars represent SEM, *significant after Bonferroni-Holm correction.

Oxidative Stress

Western blots were performed on whole cell liver lysate and probed with an anti-4-HNE antibody took look for changes in 4-HNE adduct formation, a key indicator of lipid peroxidation resulting from ROS. No differences were observed among the diet groups (Supplementary Figure 14).

Methionine Cycle

In order to evaluate ethanol's impact on methyl donors, which may impact the transmethylation pathway as well as the ability to regenerate the ROS scavenger glutathione, total hepatic SAMe and SAH were determined using HPLC (Figure 8A-B). Although the data indicate that in 22 week treated mice, lower dietary iron leads to decreases in SAMe levels and the SAMe:SAH ratio in ethanol treated mice vs pair-fed controls; the sample size was small, variance was high and the results were not statistically significant.

Glutathione

Glutathione was assessed with a spectrophotometric assay (Figure 8C-D). No changes were observed in either total or oxidized glutathione at 22 weeks of ethanol exposure.

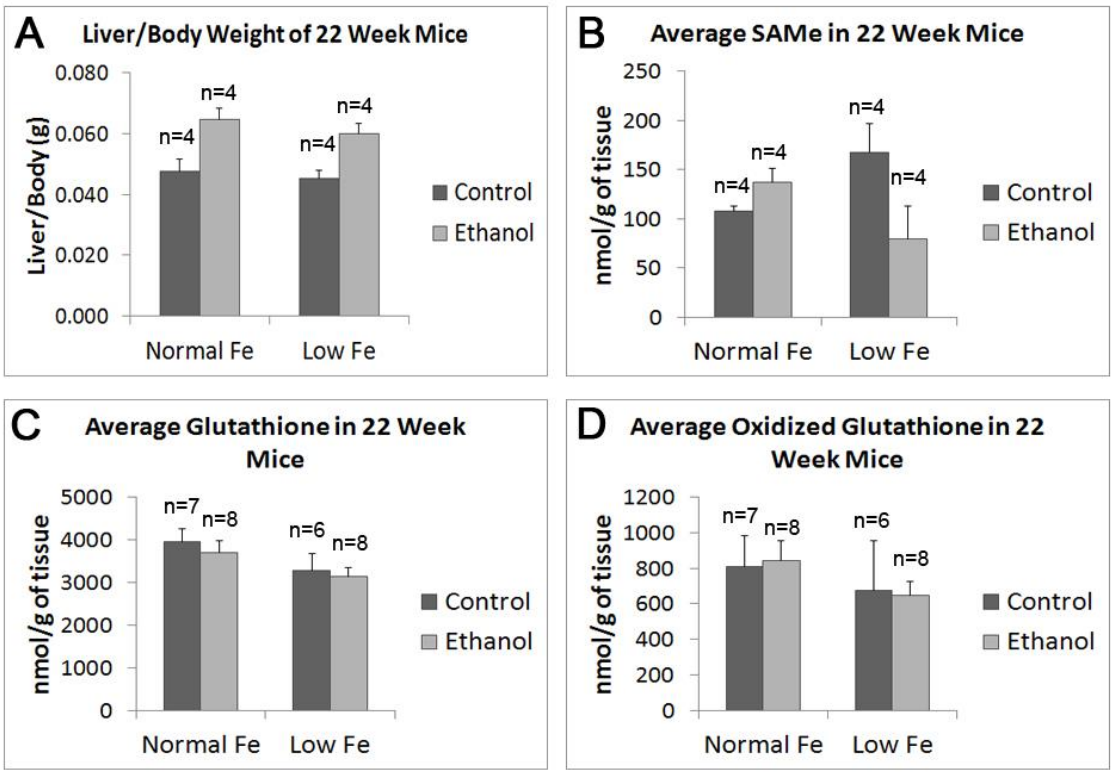


Figure 8. Hepatic SAMe and glutathione status in 22 week ethanol fed mice. (**A**) SAMe:SAH ratio in mice fed ethanol for 22 weeks. (**B**) Total hepatic SAMe. (**C**-**D**) Total hepatic glutathione content and oxidized glutathione in mice treated with ethanol for 22 weeks, respectively.

DNA Methylation

Despite no apparent changes in SAMe, we measured global DNA methylation in order to see if the steady state level of methyl donors was being maintained through reduction in downstream usage of SAMe. There were no significant differences in DNA methylation using LUMA between diet groups in mice exposed to ethanol treatment for 22 weeks (Figure 9).

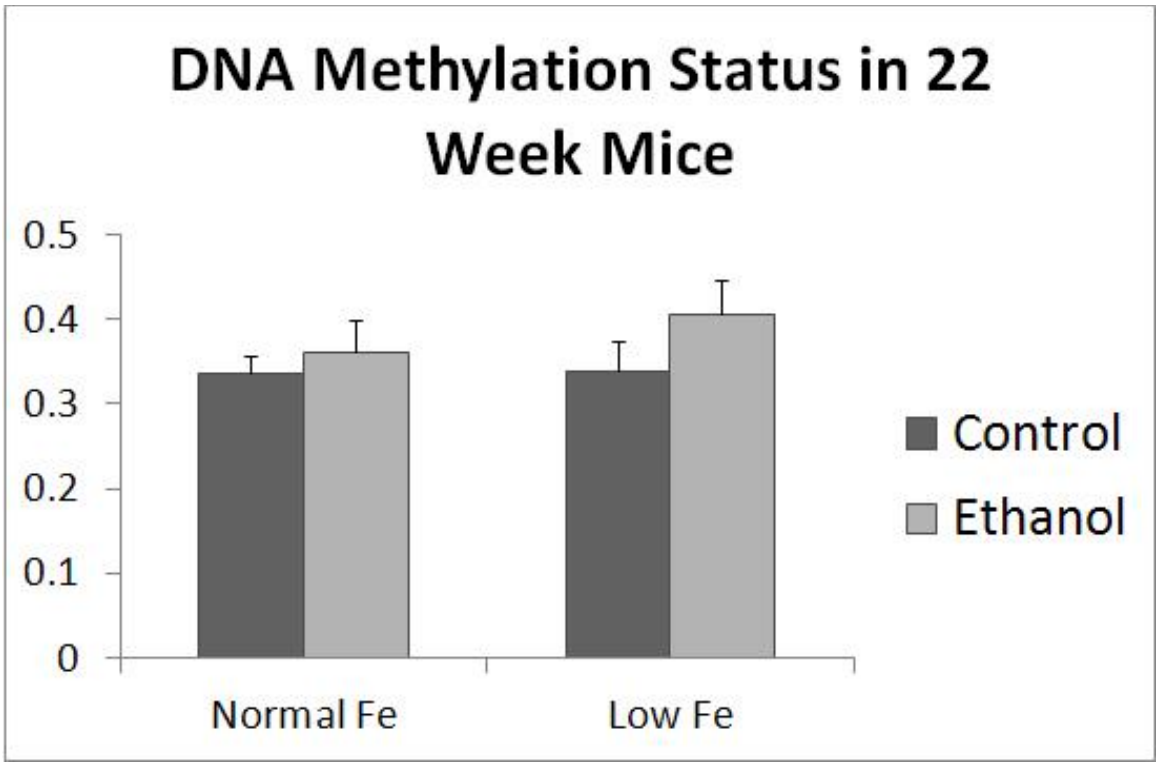


Figure 9. DNA methylation status in mice fed ethanol for 22 weeks. Global DNA methylation was measured in mice fed ethanol for 22 weeks using a LUMA assay.

Protein acetylation, NAD^+/NADH and iron protein status

Western blot data from liver tissue lysate of wave 1 mice that received ethanol treatment for 11 weeks is represented in Figure 10A, with densitometry data shown in 10B. The graphs in panel B show relative protein expression normalized to the normal iron control group. Global hepatic lysine acetylation was shown to be increased in both ethanol diets with a 62% increase in the normal iron group and a 124% ($p<0.05$) increase in the low iron group, over their respective controls. While the low iron control levels were lower than their normal iron control counterparts, the difference was not significant at the 0.05 α level. The iron responsive proteins FerL was decreased in both low iron groups, while TfR expression was elevated by a factor of 3 ($p<0.001$) in the low iron ethanol group only. The transcription factor targeting deacetylase Sirt1, was increased in both ethanol groups 71% and 184% ($p<0.05$) in the normal iron and low iron groups, respectively. Interestingly, there was a 2 fold increase ($p<0.05$) in the low iron control group. There were no significant changes observed in the tublin deacetylase, HDAC6.

NAD^+ and NADH levels were quantified in the 11 week mice as shown in Figure 12A, there were no significant changes in the ratio of NAD^+/NADH or the actual amount of NAD^+ or NADH per 1mg of protein between groups.

Western blotting of whole cell liver tissue lysate was performed on mice receiving ethanol treatment for 22 weeks in order to analyze protein expression as shown in Figure 11. Figure 11A depicts a representative western blot image of the proteins of interest including global hepatic lysine acetylation, the iron responsive proteins FerL and

TfR, and deacetylases Sirt1 and HDAC6. Densitometry was used to measure relative protein expression and normalized to the normal iron control groups that were probed on the same gel. A graphical representation of protein expression for these proteins from multiple gels is shown in Figure 11B. A 47% ($p<0.05$) increase in hepatic lysine acetylation was observed in the normal iron ethanol group and a 142% ($p<0.01$) increase in the low iron ethanol group when compared to the normal iron control. There was an 80% ($p<0.001$) reduction in hepatic FerL expression in the low iron control group over the normal iron control, however, FerL expression was found to be decreased by roughly half in the low iron control group. Conversely, there was a 197% ($p<0.05$) increase in TfR expression in the low iron ethanol group and only a 26% increase in the low iron control group. We did not find significant changes in HDAC6 or Sirt1 expression between diet groups.

Figure 12B represents NAD^+ and NADH levels in 22 week mice. There were no significant changes observed between groups with the NAD^+/NADH ratios. Interestingly, there were changes in NAD^+ and NADH amounts per 1mg of protein. Overall, there was a nearly 2 fold decrease in total NAD^+ and NADH together in both low iron groups over the normal iron control. NAD^+ levels were down by 33% ($p<0.05$) in the low iron control group and a 42% ($p<0.05$) decrease in the low iron ethanol group compared to the normal iron control. There was a similar decrease in NADH in both low iron groups of 40% ($p<0.05$) compared to the normal iron control.

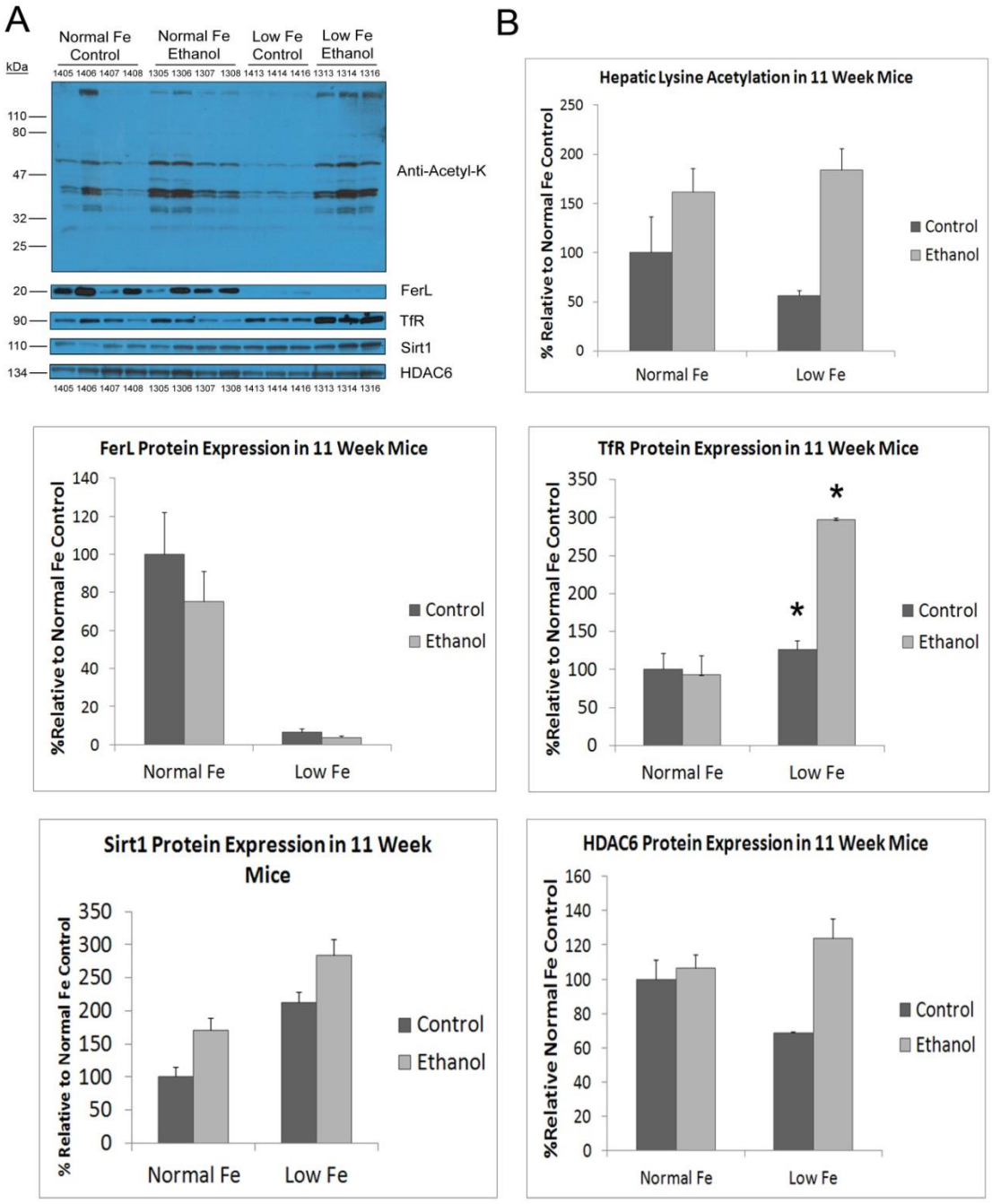


Figure 10. Hepatic lysine acetylation status and protein expression in 11 week ethanol fed mice. (**A**) Western blot image of lysine acetylation, iron responsive proteins FerL (ferritin light chain) and TfR (transferrin receptor), and lysine deacetylases Sirt1 (sirtuin) and HDAC6 (histone deacetylase) from whole cell liver tissue lysate. (**B**) Densitometry data demonstrating relative protein expression levels normalized to the normal iron control group. Error bars represent SEM, *significant after Bonferroni-Holm correction.

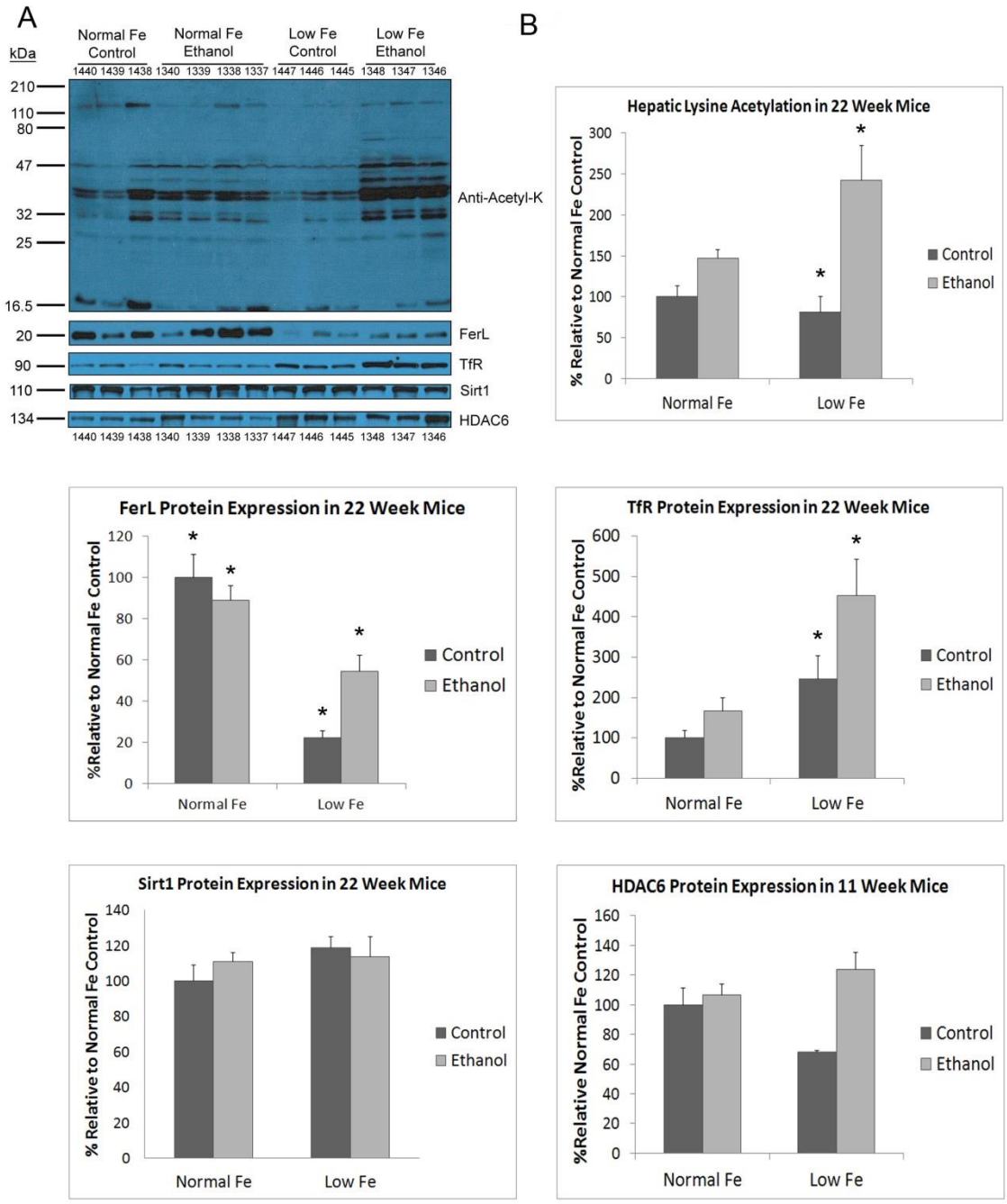
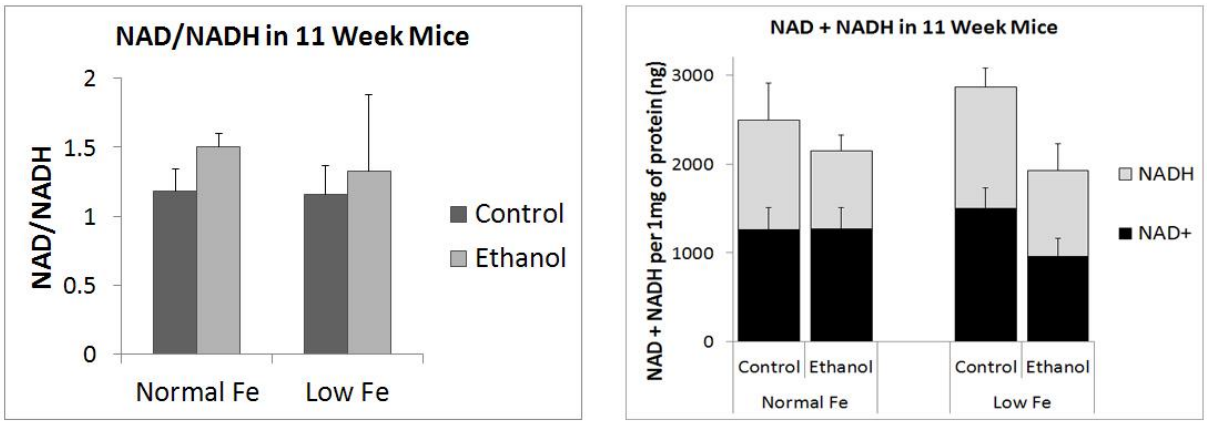


Figure 11. Hepatic lysine acetylation status and protein expression in 22 week ethanol fed mice. (**A**) Representative western blot image of lysine acetylation, iron responsive proteins FerL (ferritin light chain) and TfR (transferrin receptor), and lysine deacetylases Sirt1 (sirtuin) and HDAC6 (histone deacetylase) from whole cell liver tissue lysate. (**B**) Densitometry data demonstrating relative protein expression levels normalized to the normal iron control group. Error bars represent SEM, *significant after Bonferroni-Holm correction.

A



B

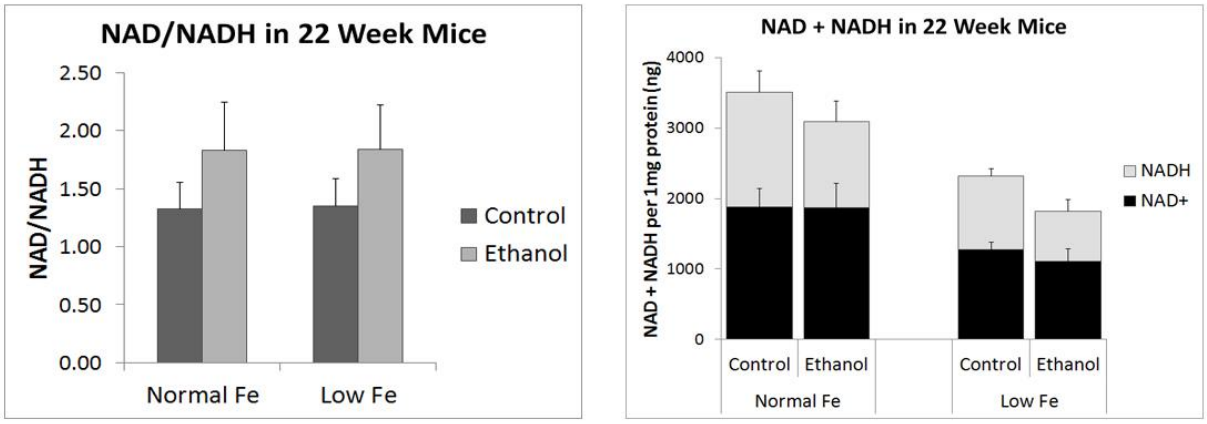


Figure 12. NAD^+ quantification. (**A**) NAD^+/NADH and total NAD+ and NADH levels in 11 week ethanol fed mice. (**B**) NAD^+/NADH and total NAD^+ and NADH levels in 22 week ethanol fed mice. Error bars represent SEM, *significant after Bonferroni-Holm correction.

2.4 Discussion

In the present study, the hypothesis that normal levels of dietary iron play a role in the impact of ethanol after chronic ethanol consumption was evaluated in mice. After 22 weeks of feeding, despite an increase in liver to body weight ratios of roughly 30%, we found no direct test result that would indicate significant oxidative stress was occurring as a result of ethanol treatment, at either normal or low levels of iron intake. Previous studies have reported significant increases in 4-HNE adducts in rats fed ethanol, with one study in particular reporting that 4-HNE adducts were amplified by increased dietary iron in the form of carbonyl iron [67, 70, 75, 76]. However, these studies have used intragastric catheters to continuously feed the animals high fat diets containing up to 49% of calories from ethanol for up to 16 weeks, enabling liver pathology to advance to the stage of cirrhosis and fibrosis. Many studies have utilized intragastric ethanol feeding to study the chronic effects of alcoholic liver disease in rats and mice. The advantage is in overcoming the animals' natural aversion to alcohol. While intragastric feeding can achieve blood alcohol concentrations (BAC) as high as 500-600mg/dL, Lieber–DeCarli liquid ethanol diets (LDL) can only reach an average of 100-160mg/dL and may be the reason advanced staged liver disease beyond steatosis is difficult to achieve [77]. Rats that were fed a LDL ethanol diet for a period of 9 months failed to produce pathology in the liver beyond steatosis, however with vitamin A supplementation combined with ethanol treatment produced necrosis, inflammation and fibrosis [78]. Indeed, the inclusion of a secondary hepatic stressor such as lipopolysaccharides, high fat diet, iron, hepatotoxins, or viral proteins appears to be a

requirement to initiate the progression of cirrhosis or hepatocellular carcinoma in the LDL feeding model [77]. The employment of LDL ethanol diet alone does, however, appear to induce CYP2E1, increased triglycerides, changes in iron homeostasis, and nutritional deficiencies [77].

In order to determine the response to differences in dietary iron content in terms of iron phenotype, we examined the expression of the iron responsive proteins TfR and FerL. As expected, the low iron diet group experienced an overall decrease in FerL expression at both time points when compared to the control groups. TfR levels were significantly increased in low iron mice when compared to the control groups only at the 22 week time point. We did not see significant changes in the normal iron group.

Of particular interest, TfR was substantially up-regulated in combination with ethanol treatment and a low iron diet at 11 weeks and 22 weeks relative to the pair fed controls. Additionally, FerL was up-regulated in the low iron ethanol group compared to the low iron control at 22 weeks, but not at 11 weeks. Previous studies have reported that LDL ethanol treatment in rats have led to increased hepatic ferritin expression with no changes in TfR [79]. This has been shown to correlate with ethanol mediated down-regulation of hepcidin, also rendering hepcidin insensitive to its normal response to increases in hepatic iron stores [3, 79]. In another report, the effect of ethanol on hepcidin was independent of any direct effects on IRP's (iron response element binding proteins), TfR or ferritin [80]. These studies generally took place over the course of a week of ethanol exposure and did not examine the expression of these iron proteins over a prolonged period of time. While TfR has been found to be increased in the hepatic tissue of patients with alcoholic liver disease [71], the use of decreased iron in

ethanol treatment is unique to this study. We speculate that the low iron diet created an iron poor systemic environment for the liver cells, leading to the strong reductions in ferritin that were observed at 11 weeks. Despite this, the levels of intracellular iron were not low enough by that point to lead to significant increases in transferrin receptor expression in the non-ethanol fed mice. After 22 weeks of treatment, however, the liver iron stores were depleted sufficiently to generate a low cellular iron condition consistent with the observed increase in transferrin receptor expression in the low iron diet group non ethanol fed mice. The increased TfR expression seen in ethanol treated low iron fed mice versus their pair fed low iron controls at both time points does not fit current models, but clearly may play a role in reports that find increased hepatic iron associated with alcohol consumption [3, 4], as increases in TfR would facilitate increased uptake of systemic iron. This result is intriguingly consisitent with the finding that, at 22 weeks, the low iron, ethanol fed mice had a strong rise in ferritin, relative to the low iron, non-ethanol pair-fed controls, as the presense of the ferritin may indicate a relative increase in iron stores between these two groups. This mechanism would also be facilitated if systemic iron uptake and availability were increased due to the impact of ethanol on hepcidin secretion, as has been reported [10, 14, 79].

4-Methylpyrazole is an inhibitor of alcohol metabolizing enzymes, and has been used to abolish the effect of ethanol on hepcidin in cell culture [80]. It might be interesting to see if 1) TfR is up-regulated in ethanol treated hepatic cells in iron deficient media; and 2) how these cells might respond if 4-methylpyrazole is introduced with ethanol treatment.

Over the last decade it has been discovered that ethanol treatment in mice leads to global hepatic lysine hyperacetylation in a wide variety of nuclear and non-nuclear proteins [57, 59]. A novel finding in our study is that a reduction in dietary iron significantly increases global hepatic lysine hyperacetylation in whole cell lysate from hepatic tissue of mice exposed to ethanol treatment for 22 weeks compared to ethanol fed mice receiving a normal iron diet. This finding led to our examination of cellular NAD^+ and NADH levels and the expression of two deacetylases known to be active in the cytosol.

One prevailing notion is that because alcohol consumption depletes NAD^+ though the oxidation of ethanol, perhaps NAD^+ dependent sirtuin activity also decreases with the diminishing supply of NAD^+ [39]. We found that the ratio of NAD^+/NADH was not impacted significantly in any of the diet groups at 11 weeks or 22 weeks. Nevertheless, we were surprised to find that the actual amounts of NAD^+ and NADH were decreased nearly two-fold in the low iron diet group for both ethanol and control mice at 22 weeks. However, this finding may not be an accurate representation of the actual cellular redox state. NAD^+ and NADH are unevenly distributed throughout different cellular compartments and can be free or protein-bound [81]. In order to obtain the ratio of free NAD^+/NADH in a specific compartment, one can measure the concentrations of oxidized and reduced reactants of a suitable near-equilibrium reaction. Traditionally, lactate/pyruvate concentrations are measured to determine free cytosolic NAD^+/NADH, while acetoacetate/β-hydroxybutyrate concentrations measure the ratio in mitochondria [82]. Similar methods have been used to detect decreases in cytoplasmic and mitochondrial NAD^+/NADH ratios in rats given intraperitoneal injections

of ethanol [83]. However, a decrease in NAD^+/NADH ratio has also been observed from direct quantification of the molecules using a lactate dehydrogenase based cycling enzyme in mice fed a LDL ethanol diet for 6 weeks consisting of 31% ethanol derived calories [84]. It might also be worth noting that the LDL diet in this study contained 44% calories derived from fat, whereas our study used 35%.

Although we did not observe a significant change in the ratio of total NAD^+/NADH, there was a reduced amount of NAD^+ in the low iron group, which also had the highest amount of acetylation in ethanol fed mice. This finding alone might support the possibility that a decrease in NAD^+ leads to decrease sirtuin activity. Sirt1 expression has been found to be down-regulated in mice on a low fat (10%) LDL ethanol diet for 4 weeks [85]. A second study reported a decrease in both protein and mRNA of Sirt1 and its target protein PGC-1α in rats fed a diet with 47% ethanol-derived calories, and 32% fat in the form of long chain triglycerides [64]. Levels were restored to normal when the fat content was replaced with medium chain triglycerides. The reverse has been reported where Sirt1 protein expression is increased in mice receiving LDL ethanol treatment with high saturated fats for a month [86], and in rats intragastrically fed ethanol diet for the same amount of time [69]. Our study did not find significant differences in Sirt1 expression at 22 weeks of ethanol feeding. At 11 weeks Sirt1 was increased in the ethanol fed normal iron and low iron control group, and further increased in low iron ethanol fed mice. This increase may, in part, be influenced by the fat content in our diet since previous studies have indicated changes related directly to the fat content combined with ethanol treatment. It may also be the result of a yet to be defined feedback mechanism related to alcohol-induced lysine acetylation

that attenuates over more prolonged ethanol exposure. Notably, at 11 weeks when Sirt1 expression was increased in ethanol fed mice, a significant increase in lysine acetylation in low iron ethanol fed mice vs normal iron ethanol fed mice was not apparent. After 22 weeks, when Sirt1 levels were equivalent between the groups, low iron ethanol fed mice had significantly more acetylation than normal iron ethanol fed mice. Acetylation status of PGC-1α, a 113kDa protein that regulates gluconeogenesis and a substrate of Sirt1, is another commonly used indicator of Sirt1 activity [85]. However, the protein was not apparent on the lysine acetylation western blots.

In order to determine if there were changes in deacetylase activity that are not dependent on NAD^+, we chose to examine HDAC6 expression. HDAC6 is a microtubule deacetylase and is the only exclusively cytosolic HDAC found in the liver [39]. Tubulin hyperacetylation is a feature that is known to occur with ethanol treatment in WIF-B and rats [58, 60]. In ethanol treated hepatic cells, HDAC6 expression has been shown to decrease slightly [58]. Additionally, HDAC6 binding to microtubules is impaired, presumably, due to ethanol induced modification of tubulin mediated by acetaldehyde [58]. To our knowledge, HDAC6 expression has not been examined *in vivo* with long term ethanol treatment. In the present study we did not find an appreciable change in HDAC6 expression at 11 weeks or 22 weeks, supporting the idea that HDAC6 activity may be impaired rather than its expression, or that this may not significantly impact overall protein acetylation which results from ethanol treatment.

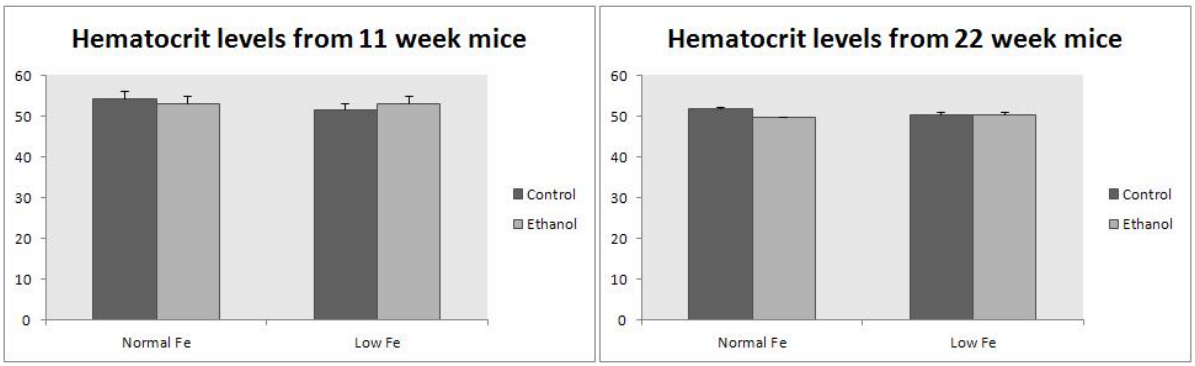
Despite our attempts to detail the mechanism behind ethanol induced acetylation, it remains elusive, and to date there has been nothing in the literature to suggest that iron inhibits lysine acetylation or is a requirement for lysine deacetylation.

Further studies are needed to determine why the reduction of dietary iron leads to an increase global lysine acetylation and a reduction in the amount of NAD^+ and NADH in the context of chronic ethanol consumption. It can be deduced that the level of iron by itself has no significant effect on acetylation status since the presence of ethanol was a requirement for hyperacetylation. It has recently been discovered that an enzyme required in the earliest steps of NAD *de novo* biosynthesis, quinolinate synthetase is an iron-sulfur cluster containing enzyme [87, 88]. It might interesting to perform an assay on quinolinate synthetase activity on hepatic tissue or cells starved of iron. Nevertheless, while it is tempting to blame the phenomenon of hyperacetylation on the decrease in NAD^+, the required cofactor for sirtuin deacetylase activity, we found no increase in acetylation of the low iron non-ethanol fed mice. If the increase in acetylation due to low iron stems from a decrease in sirtuin activity, then it seems to be only detectable against a background of high acetylation. Future studies could also include an assessment of acetyltransferase activity in the liver with low iron and ethanol. Determining the amount of acetate in the liver could also provide useful information since it is required for acetyl group donation and there is evidence in histones that non-enzymatic lysine acetylation can occur and this possibility might exist within the mitochondria as well since there has been no mitochondrial acetyltransferase discovered to date [65]. Therefore, a simple explanation could be that low iron somehow has an impact on acetate processing, perhaps leading to increased acetate-protein interaction which in turn leads to increased acetylation.

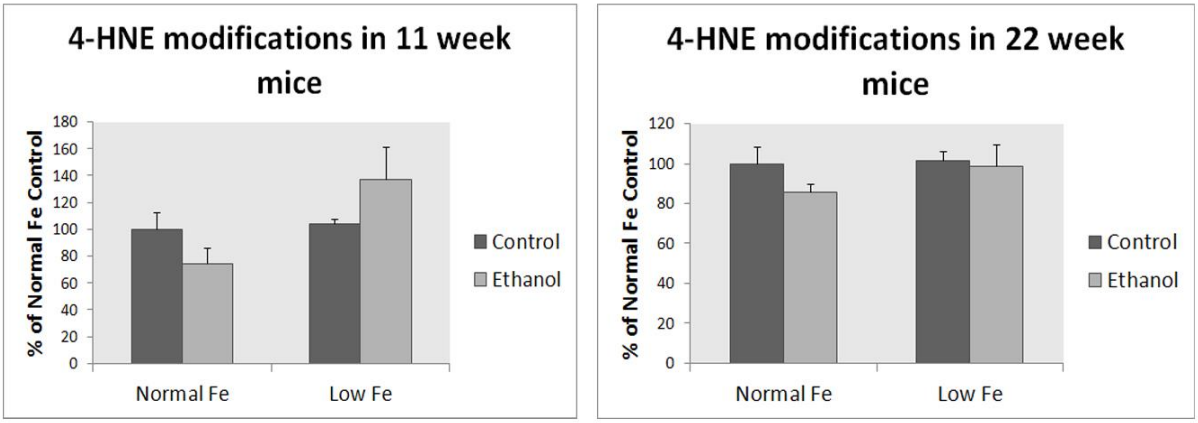
In the present study we found that decreasing dietary iron has surprising effects on iron homeostasis, NAD levels and lysine acetylation when compared to normal

dietary iron intake. Measurements of oxidative stress and methylation status were unchanged between ethanol and control groups, with no apparent association to dietary iron. Conversely, after 22 weeks of treatment, low levels of dietary iron were associated with decreased total nicotinamide NAD^+/NADH levels, and increased ethanol-derived lysine hyperacetylation. Investigation of the expression of two protein deacetylases, Sirt1 and HDAC6 did not indicate the expression of these proteins were responsible for the observed impact of the low iron diet on protein hyperacetylation. Low iron diets predictably led to decreases in ferritin expression after both 11 and 22 weeks, while TfR expression was only increased at the 22 week time point. Ethanol consumption led to increased TfR expression over the low iron pair-fed controls at both time points. Additionally, ferritin expression was increased in the low iron ethanol 22 week group relative to the low iron pair fed controls, indicating normal iron homeostatic mechanisms were disrupted by ethanol in a low iron setting. The results of this study suggest that the trend for chronic alcohol consumers to increase hepatic stores of iron may involve alterations in transferrin receptor regulation which would interfere with dietary management of iron loading using a strategy of reduced iron consumption. More research in this area is needed to fully interpret the observations relating to ethanol consumption and iron deficiency.

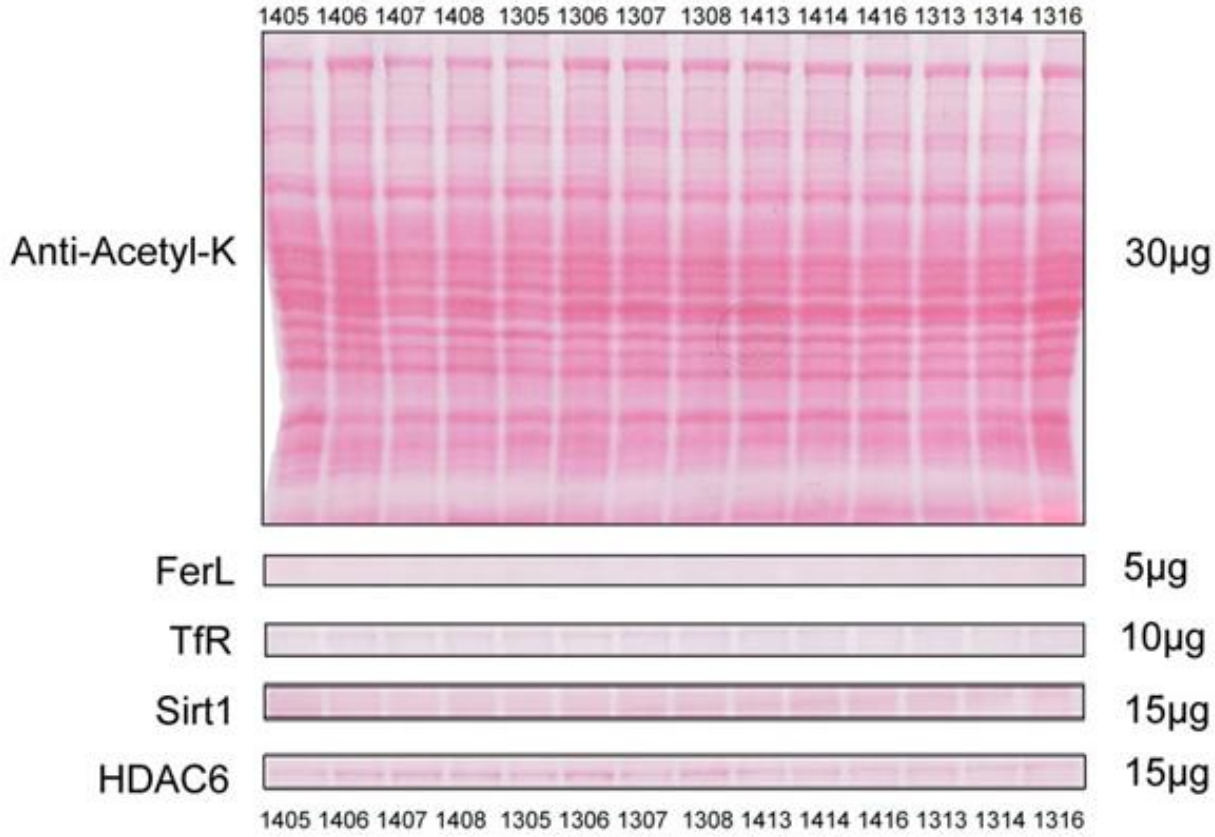
2.5 Supplementary Data



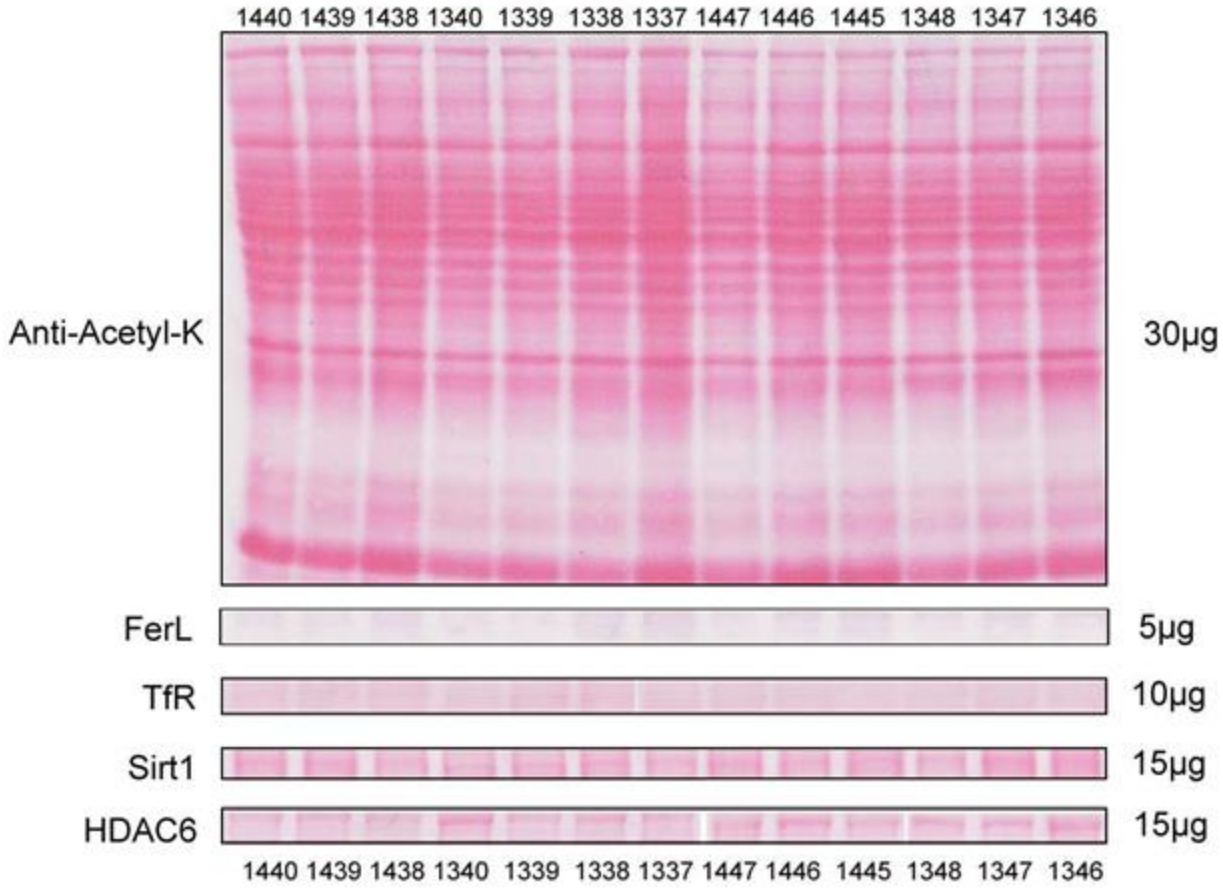
Supplementary Figure 13. Hematocrit data from mice fed ethanol for 11 and 22 weeks. Hematocrit data was taken from the mice at the time of sacrifice. No significant changes were observed among diet groups.



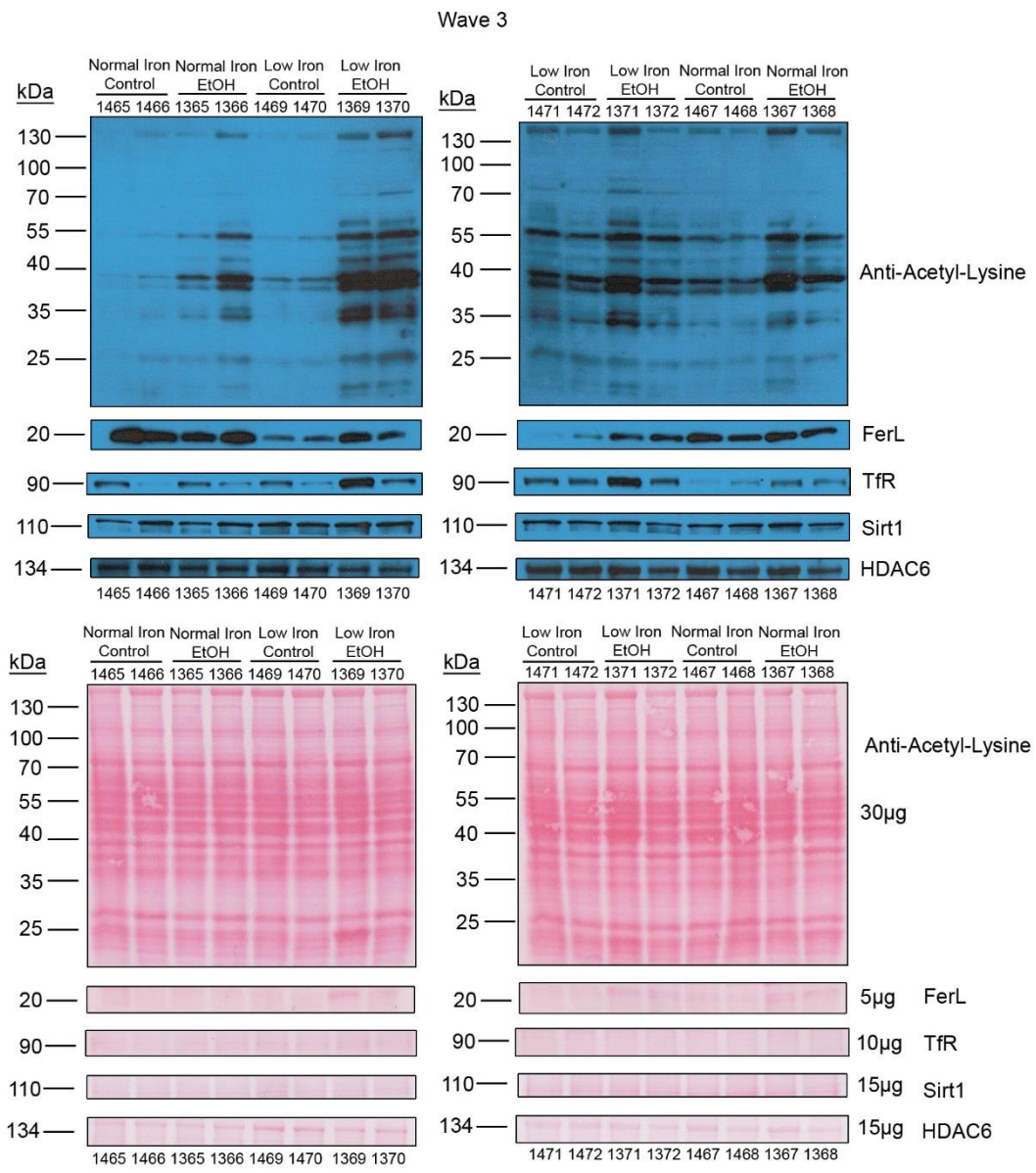
Supplementary Figure 14. Densitometry data of 4-HNE modifications in mice fed ethanol for 11 and 22 weeks normalized to the normal iron control group. 4-HNE adducts were measured by western blotting using an anti-4-HNE antibody. No significant changes were observed among diet groups.



Supplementary Figure 15. Ponceau-S stain of membranes used for western blotting of 11 week ethanol fed mice.



Supplementary Figure 16. Ponceau-S stain of membranes used for western blotting of 22 week mice.



Supplementary Figure 17. Western blots and ponceau-S stains of 22 week mice.

REFERENCES

1. Mokdad, A.H., et al., *Actual causes of death in the United States, 2000.* JAMA: The Journal of the American Medical Association, 2004. **291**(10): p. 1238-1245.

2. Rehm, J., et al., *Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders.* The Lancet, 2009. **373**(9682): p. 2223-2233.

3. Harrison-Findik, D.D., *Role of alcohol in the regulation of iron metabolism.* World Journal of Gastroenterology, 2007. **13**(37): p. 4925-30.

4. Ioannou, G.N., et al., *The effect of alcohol consumption on the prevalence of iron overload, iron deficiency, and iron deficiency anemia.* Gastroenterology, 2004. **126**(5): p. 1293-1301.

5. Takami, T. and I. Sakaida, *Iron regulation by hepatocytes and free radicals.* Journal of Clinical Biochemistry and Nutrition, 2011. **48**(2): p. 103-6.

6. Zakhari, S., *Overview: how is alcohol metabolized by the body?* Alcohol Research & Health: The Journal of the National Institute on Alcohol Abuse and Alcoholism, 2006. **29**(4): p. 245.

7. Fleming, R.E. and B.R. Bacon, *Orchestration of iron homeostasis.* New England Journal of Medicine, 2005. **352**(17): p. 1741-1744.

8. Björn-Rasmussen, E., et al., *Food iron absorption in man: Applications of the two-pool extrinsic tag method to measure heme and nonheme iron absorption from the whole diet.* Journal of Clinical Investigation, 1974. **53**(1): p. 247.

9. Hallberg, L., et al., *Dietary heme iron absorption: a discussion of possible mechanisms for the absorption-promoting effect of meat and for the regulation of iron absorption.* Scandinavian Journal of Gastroenterology, 1979. **14**(7): p. 769-779.

10. Ganz, T., *Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation.* Blood, 2003. **102**(3): p. 783-788.

11. Wang, J. and K. Pantopoulos, *Regulation of cellular iron metabolism.* Biochemical Journal, 2011. **434**(Pt 3): p. 365.

12. Stäubli, A. and U.A. Boelsterli, *The labile iron pool in hepatocytes: prooxidant-induced increase in free iron precedes oxidative cell injury.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 1998. **274**(6): p. G1031-G1037.

13. Arruda, L.F., et al., *Dietary iron concentration may influence aging process by altering oxidative stress in tissues of adult rats.* PloS One, 2013. **8**(4): p. e61058.

14. Harrison-Findik, D.D., *Is the iron regulatory hormone hepcidin a risk factor for alcoholic liver disease?* World Journal of Gastroenterology, 2009. **15**(10): p. 1186.

15. Manzo-Avalos, S. and A. Saavedra-Molina, *Cellular and mitochondrial effects of alcohol consumption.* International Journal of Environmental Research and Public Health, 2010. **7**(12): p. 4281-4304.

16. Guarente, L., *Sirtuins as potential targets for metabolic syndrome.* Nature, 2006. **444**(7121): p. 868-874.

17. Scott, M.D., et al., *NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes.* Blood, 1991. **77**(9): p. 2059-2064.

18. Guo, R. and J. Ren, *Alcohol and acetaldehyde in public health: from marvel to menace.* International Journal of Environmental Research and Public Health, 2010. **7**(4): p. 1285-1301.

19. Berg, J.M., J.L. Tymoczko, and L. Stryer, *Ethanol alters energy metabolism in the liver.* Biochemistry, 5th edition, 2002.

20. Halperin, M., et al., *Metabolic acidosis in the alcoholic: a pathophysiologic approach.* Metabolism, 1983. **32**(3): p. 308-315.

21. Eaton, S., C. Record, and K. Bartlett, *Multiple biochemical effects in the pathogenesis of alcoholic fatty liver.* European Journal of Clinical Investigation, 1997. **27**(9): p. 719-722.

22. Leandro, G., et al., *Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data.* Gastroenterology, 2006. **130**(6): p. 1636-1642.

23. Mato, J., et al., *S-adenosylmethionine synthesis: Molecular mechanisms and clinical implications.* Pharmacology & Therapeutics, 1997. **73**(3): p. 265-280.

24. Osna, N.A., *Role of transmethylation reactions in alcoholic liver disease.* World Journal of Gastroenterology, 2007. **13**(37): p. 4947-4954.

25. Barak, A.J., et al., *Effects of prolonged ethanol feeding on methionine metabolism in rat liver.* Biochemistry and Cell Biology, 1987. **65**(3): p. 230-233.

26. Lu, S.C., et al., *Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2000. **279**(1): p. G178-G185.

27. Lindenbaum, J. and C.S. Lieber, *Alcohol-induced malabsorption of vitamin B12 in man.* Nature, 1969. **224**(5221): p. 806.

28. Bailey, L.B. and J.F. Gregory, 3rd, *Folate metabolism and requirements.* The Journal of Nutrition, 1999. **129**(4): p. 779-82.

29. Yang, X. and E. Seto, *HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention.* Oncogene, 2007. **26**(37): p. 5310-5318.

30. Gu, W. and R.G. Roeder, *Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain.* Cell, 1997. **90**(4): p. 595-606.

31. Kim, S.C., et al., *Substrate and functional diversity of lysine acetylation revealed by a proteomics survey.* Molecular Cell, 2006. **23**(4): p. 607.

32. Zhao, S., et al., *Regulation of cellular metabolism by protein lysine acetylation.* Science, 2010. **327**(5968): p. 1000-1004.

33. Choudhary, C., et al., *Lysine acetylation targets protein complexes and co-regulates major cellular functions.* Science Signaling, 2009. **325**(5942): p. 834.

34. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications.* Cell Research, 2011. **21**(3): p. 381-395.

35. Kouzarides, T., *Acetylation: a regulatory modification to rival phosphorylation?* Science Signaling, 2000. **19**(6): p. 1176.

36. Eddé, B., et al., *Posttranslational modifications of tubulin in cultured mouse brain neurons and astroglia.* Biology of the Cell, 1989. **65**(2): p. 109-117.

37. Maruta, H., K. Greer, and J.L. Rosenbaum, *The acetylation of alpha-tubulin and its relationship to the assembly and disassembly of microtubules.* The Journal of Cell Biology, 1986. **103**(2): p. 571-579.

38. Close, P., et al., *The emerging role of lysine acetylation of non-nuclear proteins.* Cellular and Molecular Life Sciences, 2010. **67**(8): p. 1255-1264.

39. Shepard, B.D. and P.L. Tuma, *Alcohol-induced protein hyperacetylation: mechanisms and consequences.* World Journal of Gastroenterology, 2009. **15**(10): p. 1219.

40. Friedmann, D.R. and R. Marmorstein, *Structure and mechanism of non-histone protein acetyltransferase enzymes.* FEBS Journal, 2013.

41. Xing, S. and Y. Poirier, *The protein acetylome and the regulation of metabolism.* Trends in Plant Science, 2012.

42. Yang, C., et al., *Autoacetylation of the MYST lysine acetyltransferase MOF protein.* Journal of Biological Chemistry, 2012. **287**(42): p. 34917-26.

43. Yuan, H., et al., *MYST protein acetyltransferase activity requires active site lysine autoacetylation.* The EMBO Journal, 2011. **31**(1): p. 58-70.

44. Albaugh, B.N., et al., *Autoacetylation of the histone acetyltransferase Rtt109.* Journal of Biological Chemistry, 2011. **286**(28): p. 24694-24701.

45. Blanco-García, N., et al., *Autoacetylation regulates P/CAF nuclear localization.* Journal of Biological Chemistry, 2009. **284**(3): p. 1343-1352.

46. Kalebic, N., et al., *Tubulin acetyltransferase alphaTAT1 destabilizes microtubules independently of its acetylation activity.* Molecular Cell Biology, 2013. **33**(6): p. 1114-23.

47. Nambi, S., et al., *Cyclic AMP-induced conformational changes in mycobacterial protein acetyltransferases.* Journal of Biological Chemistry, 2012. **287**(22): p. 18115-18129.

48. Lee, H.J., et al., *Cyclic AMP regulation of protein lysine acetylation in Mycobacterium tuberculosis.* Nature Structural & Molecular Biology, 2012.

49. Aka, J.A., G.-W. Kim, and X.-J. Yang, *K-acetylation and its enzymes: Overview and new developments.* Histone Deacetylases: The Biology and Clinical Implication, 2011. **206**: p. 1.

50. Zhang, Y., et al., *Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally.* Molecular Cell Biology, 2008. **28**(5): p. 1688-701.

51. Cohen, H.Y., et al., *Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase.* Science Signaling, 2004. **305**(5682): p. 390.

52. Rodgers, J.T., et al., *Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1.* Nature, 2005. **434**(7029): p. 113-118.

53. Luo, J., et al., *Negative control of p53 by Sir2alpha promotes cell survival under stress.* Cell, 2001. **107**(2): p. 137-48.

54. North, B.J., et al., *The Human Sir2 Ortholog, SIRT2, Is an NAD+-Dependent Tubulin Deacetylase.* Molecular Cell, 2003. **11**(2): p. 437-444.

55. Michishita, E., et al., *SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin.* Nature, 2008. **452**(7186): p. 492-496.

56. Ford, E., et al., *Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription.* Genes & Development, 2006. **20**(9): p. 1075-1080.

57. Picklo, M.J., *Ethanol intoxication increases hepatic N-lysyl protein acetylation.* Biochemical and Biophysical Research Communications, 2008. **376**(3): p. 615-619.

58. Shepard, B.D., et al., *Alcohol-induced alterations in hepatic microtubule dynamics can be explained by impaired histone deacetylase 6 function.* Hepatology, 2008. **48**(5): p. 1671-9.

59. Shepard, B.D., D.J. Tuma, and P.L. Tuma, *Chronic ethanol consumption induces global hepatic protein hyperacetylation.* Alcoholism: Clinical and Experimental Research, 2010. **34**(2): p. 280-291.

60. Kannarkat, G.T., D.J. Tuma, and P.L. Tuma, *Microtubules are more stable and more highly acetylated in ethanol-treated hepatic cells.* Journal of Hepatology, 2006. **44**(5): p. 963-70.

61. Park, P.-H., R. Miller, and S.D. Shukla, *Acetylation of histone H3 at lysine 9 by ethanol in rat hepatocytes.* Biochemical and Biophysical Research Communications, 2003. **306**(2): p. 501-504.

62. Joseph, R.A., et al., *Microtubule acetylation and stability may explain alcohol-induced alterations in hepatic protein trafficking.* Hepatology, 2008. **47**(5): p. 1745-1753.

63. Lieber, C.S., et al., *Alcohol alters hepatic FoxO1, p53, and mitochondrial SIRT5 deacetylation function.* Biochemical and Biophysical Research Communications, 2008. **373**(2): p. 246-252.

64. Lieber, C.S., et al., *Effect of chronic alcohol consumption on hepatic SIRT1 and PGC-1alpha in rats.* Biochemical and Biophysical Research Communications, 2008. **370**(1): p. 44-8.

65. Wagner, G.R. and R.M. Payne, *Mitochondrial acetylation and diseases of aging.* Journal of Aging Research, 2011. **2011**.

66. Cimen, H., et al., *Regulation of succinate dehydrogenase activity by SIRT3 in mammalian mitochondria.* Biochemistry, 2010. **49**(2): p. 304-11.

67. Kamimura, S., et al., *Increased 4-hydroxynonenal levels in experimental alcoholic liver disease: association of lipid peroxidation with liver fibrogenesis.* Hepatology, 1992. **16**(2): p. 448-53.

68. Lertratanangkoon, K., et al., *Alterations of DNA methylation by glutathione depletion.* Cancer Letters, 1997. **120**(2): p. 149-156.

69. Oliva, J., et al., *Sirt1 is involved in energy metabolism: the role of chronic ethanol feeding and resveratrol.* Experimental and Molecular Pathology, 2008. **85**(3): p. 155-159.

70. Tsukamoto, H., et al., *Experimental liver cirrhosis induced by alcohol and iron.* Journal of Clinical Investigation, 1995. **96**(1): p. 620.

71. Suzuki, Y., et al., *Up-regulation of transferrin receptor expression in hepatocytes by habitual alcohol drinking is implicated in hepatic iron overload in alcoholic liver disease.* Alcoholism: Clinical and Experimental Research, 2002. **26**(s1): p. 26s-31s.

72. Pietrangelo, A., *Hereditary hemochromatosis—a new look at an old disease.* New England Journal of Medicine, 2004. **350**(23): p. 2383-2397.

73. LIEBER, C.S. and L.M. DECARLI, *Liquid diet technique of ethanol administration: 1989 update.* Alcohol and Alcoholism, 1989. **24**(3): p. 197-211.

74. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-685.

75. Li, C., et al., *Acetaldehyde-modified and 4-hydroxynonenal-modified proteins in the livers of rats with alcoholic liver disease.* Hepatology, 1997. **26**(3): p. 650-657.

76. Sampey, B.P., et al., *Immunohistochemical characterization of hepatic malondialdehyde and 4-hydroxynonenal modified proteins during early stages of ethanol-induced liver injury.* Alcoholism: Clinical and Experimental Research, 2003. **27**(6): p. 1015-1022.

77. Brandon-Warner, E., et al., *Rodent models of alcoholic liver disease: Of mice and men.* Alcohol, 2012.

78. Leo, M.A. and C.S. Lieber, *Hepatic fibrosis after long-term administration of ethanol and moderate vitamin A supplementation in the rat.* Hepatology, 1983. **3**(1): p. 1-11.

79. Harrison-Findik, D.D., et al., *Iron-mediated regulation of liver hepcidin expression in rats and mice is abolished by alcohol.* Hepatology, 2007. **46**(6): p. 1979-85.

80. Harrison-Findik, D.D., et al., *Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression.* Journal of Biological Chemistry, 2006. **281**(32): p. 22974-22982.

81. Canelas, A.B., W.M. van Gulik, and J.J. Heijnen, *Determination of the cytosolic free NAD/NADH ratio in Saccharomyces cerevisiae under steady-state and highly dynamic conditions.* Biotechnology and Bioengineering, 2008. **100**(4): p. 734-743.

82. Williamson, D., P. Lund, and H. Krebs, *The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver.* Journal of Biochemistry, 1967. **103**: p. 514-527.

83. Veech, R., R. Guynn, and D. Veloso, *The time-course of the effects of ethanol on the redox and phosphorylation states of rat liver.* Journal of Biochemistry, 1972. **127**: p. 387-397.

84. Fritz, K.S., et al., *4-Hydroxynonenal inhibits SIRT3 via thiol-specific modification.* Chemical Research in Toxicology, 2011. **24**(5): p. 651-662.

85. You, M., et al., *Involvement of mammalian sirtuin 1 in the action of ethanol in the liver.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2008. **294**(4): p. G892-G898.

86. You, M., et al., *Mammalian sirtuin 1 is involved in the protective action of dietary saturated fat against alcoholic fatty liver in mice.* The Journal of Nutrition, 2008. **138**(3): p. 497-501.

87. Ollagnier-de Choudens, S., et al., *Quinolinate synthetase, an iron-sulfur enzyme in NAD biosynthesis.* FEBS Letters, 2005. **579**(17): p. 3737-43.

88. Belenky, P., K.L. Bogan, and C. Brenner, *NAD+ metabolism in health and disease.* Trends in Biochemical Sciences, 2007. **32**(1): p. 12-9.

Appendix A: Letter from Institutional Research Board

MARSHALL UNIVERSITY.
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Office of Research Integrity

May 28, 2013

Jesse Thorton
6288 Beech Drive Apt. 7
Huntington, WV 25705

Dear Jesse:

This letter is in response to the submitted thesis abstract titled "Role of Iron in Ethanol Derived Hepatic Stress." After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #427. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director